PUBLICATIONS SUBMITTED IN SUPPORT OF AN APPLICATION BY G. C. ASHTON, B. Sc., PH.D., FOR THE DEGREE OF DOCTOR OF SCIENCE.

The 41 publications submitted are grouped in five sections, representing different areas of interest. Within each section the presentation is mainly, but not completely, chronological.

Section A: Papers on cattle transferrins.

Section B: Papers on other genetic systems in cattle.

Section C: Papers on protein and enzyme systems in species other than cattle.

Section D: Papers on comparative aspects of cattle physiology.

Section E: Papers on analytical methods (prior to 1956).

SECTION "A": CATTLE TRANSFERRINS

Paper No.	Title	Describes: -
1	Serum protein differences in cattle by starch gel electrophoresis. (1957)	-the first published account of genetic differences in the serum proteins of an animal other than man.
2	Genetics of β-globulin polymorphism in British cattle. (1958)	-first account of the genetics of transferrins in cattle.
3	β-globulin alleles in some zebu cattle. (1959)	-two further alleles at the cattle transferrin locus.
4	Serum transferrin D alleles in Australian cattle. (1965)	-recognition of two transferrin D alleles.
5	β-globulin polymorphism and early foetal mortality in cattle. (1959)	-disturbance of segregation ratios.
6	β-globulin type and fertility in artificially bred dairy cattle. (1961)	-first account of effect of transferrin genotype on fertility.
7	β-globulin type, fertility and embryonic mortality in cattle. (1962)	-extension of data in paper No. 6.
8	β-globulin polymorphism and economic factors in dairy cattle. (1960)	-first description of an association between transferrin type and milk yield.
9	Transferrin (β-globulin) type and milk and butterfat production in dairy cows. (1964)	-further data and extension of paper No. 8.
10	Transferrin and post-albumin polymorphism in E. African cattle. (1965)	-evidence for heterozygote superiority.
11	Cattle serum transferrins: a balanced polymorphism. (1965)	-evidence that cattle transferrins represent a balanced polymorphism.

(*Reprinted from Nature, Vol.* 180, *pp.* 917–919, *Nov.* 2, 1957)

Serum Protein Differences in Cattle by Starch Gel Electrophoresis

GENETICAL differences obtained with cattle serum proteins by starch gel electrophoresis have been reported briefly[1]. This communication gives details of the distribution of the phenotypes, and some mating results.

The apparatus employed is similar to that of Smithies[2], except that platinum electrodes are used and all four vessels are filled with electrolyte (42 gm./l. disodium hydrogen phosphate adjusted to *p*H 7·8 with saturated potassium dihydrogen phosphate). Starch gels are prepared at 15 per cent w/v from acid-hydrolysed potato starch (Hopkins and Williams) and a 1-in-50 dilution of electrolyte. The serum samples are inserted into the gels on pieces of Whatman *E*17 filter paper, 1·3 cm. × 0·6 cm.; three

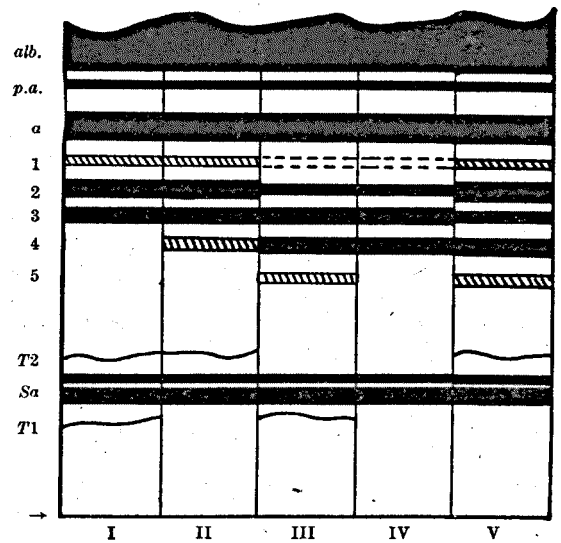


Fig. 1. Diagrammatic representation of cattle serum protein patterns by one-dimensional starch gel electrophoresis. Only the anodic side of the gel is shown. Arrow indicates position of sample insertion. I–V refer to β_s-globulin patterns, types I–V; *alb.*, part of albumin band; *p.a.*, post-albumin band (components 21 and 22, Fig. 2); 1–5, β_1-globulins; *Sa*, slow α-globulin bands (components 9 and 10, Fig. 2); *T*1 and *T*2, 'thread proteins' (component 18, Fig. 2, *T*2). Solid bands, intense staining; cross-hatched, medium staining; dotted bands, faint or absent

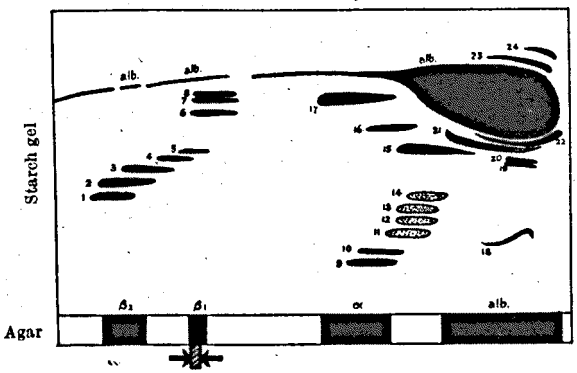


Fig. 2. Diagram prepared from epidiascope projection of starch gel after two-dimensional electrophoresis of type II serum with a T2 'thread protein'. The position of the zones separated in the initial agar electrophoresis is shown at the bottom of the diagram; the three γ-globulins have been cut off. Only the anodic side of the starch gel is shown. Arrows indicate position of original sample insertion in agar. *Alb.*, albumin; α, α-globulin; β_1 and β_{11}, β-globulin zones in agar. Components 11–14 are usually faint with poorly defined outlines

serum samples can then be run side-by-side in one 4-cm. wide gel. The current density used is 5 m.amp./cm. width of gel for gels 25 cm. long (approximately 250 V. across the electrodes). The pieces of filter paper are removed after $1\frac{1}{2}$ hr.; after 4 hr. the trailing edge of the albumin has usually migrated about 4 cm.

Examples of the patterns obtained are shown diagrammatically in Fig. 1. The nature of the components in one-dimensional starch gel electrophoresis has been determined by two-dimensional

Table 1. Distribution of β_2-Globulin Phenotypes with respect to Breed (Bulls only)

Breed	β_2-globulin phenotypes					Total No. of bulls
	I	II	III	IV	V	
Friesians	33	48	4	32	3	120
Shorthorns	16	29	1	8	2	56
Ayrshires	4	14	5	15	2	40
Herefords	8	11	2	15	nil	36
Aberdeen Angus	4	3	nil	2	1	10
Guernsey	6	10	nil	8	nil	24
Jersey	10	4	nil	3	nil	17
South Devon	1	4	nil	9	nil	14
Devon	4	4	nil	9	nil	17
Red Poll	2	4	3	nil	1	10
Lincoln Red	1	3	nil	nil	2	6
Welsh Black	nil	1	1	nil	nil	2
						352

Table 2. MATING RESULTS: MIXED BREEDS

Phenotype of parents	Phenotypes of offspring				
	I	II	III	IV	V
I × I	6	—	—	—	—
I × II	15	11	—	—	—
I × III	1	1	—	—	5
I × IV	2	7		1	—
I × V	5		—	—	2
II × II	4	4	—	5	—
II × III	—	—	—	—	2
II × IV	2	8	—	8	—
II × V	2	2	4	—	2
III × III	—	—	7	2	
III × IV	—	—	9	11	—
III × V	1	1	4	1	—
IV × IV	—	—	—	8	—
IV × V	—	3	1	—	—
V × V	1	—	—	—	2

Note: The results shown were obtained from single offspring born to 150 selected cows mated to one of 33 bulls.

electrophoresis[8], first in agar and then in starch gel (Fig. 2). The β_2-globulin zone gives four, five or six components depending on the serum type, the various combinations of these components allowing recognition of five β_2-globulin phenotypes (I–V, Fig. 1). Table 1 shows the distribution of these five phenotypes among 352 bulls at the Milk Marketing Board Cattle Breeding Centres.

The β_2-globulin phenotype of an individual animal appears to remain constant, no change being observed in individual cattle on re-sampling over a period of twelve months. Prolonged storage of serum at − 15° C. has no effect on the β_2-globulin pattern obtained. Examination of forty-two pairs of monozygous twin sera showed that in each case both members of the pair gave the same β_2-globulin pattern, suggesting genetical control. Results from 150 matings are presented in Table 2.

It seems possible from the results available at present that this system is controlled by five pairs of linked genes, so that each of the individual β_2-globulins is seen in the presence of a single or double dose of one allele of the appropriate pair, but is absent in the presence of a double dose of the other allele.

The 'thread proteins' also seem to be under genetical control, resulting in four phenotypes (Fig. 1) independent of the β_2-globulin phenotypes. However, the results obtained with this system are not reproducible, and further work is required to elucidate the mechanism.

I thank Mr. G. F. Smith, chief veterinary officer of the Milk Marketing Board, for making available

bull blood; also Drs. Hall and Jamieson, of the Animal Breeding Research Organization, Edinburgh, for supplying the monozygous twin sera, and Mr. A. L. Ogden, of this Station, for collecting blood samples in Norfolk and Suffolk.

G. C. Ashton

Animal Health Trust,
Farm Livestock Research Station,
Stock, Essex.
July 23.

[1] Ashton, G. C., *Vet. Rec.*, 69, 803 (1957).
[2] Smithies, O., *Biochem. J.*, 61, 629 (1955).
[3] Smithies, O., and Poulik, M., *Nature*, 177, 1033 (1956).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(*Reprinted from Nature, Vol.* 182, *pp.* 370–372, *Aug.* 9, 1958)

GENETICS OF BETA-GLOBULIN POLYMORPHISM IN BRITISH CATTLE

By G. C. ASHTON

The Animal Health Trust, Farm Livestock Research Station, Stock, Essex

IN a preliminary communication five serum β-globulin phenotypes of cattle, identified by starch gel electrophoresis, were described[1]. A sixth phenotype has now been found, and as a result it is possible to propose a genetic mechanism for the system.

The six phenotypes are shown in Fig. 1 together with the postulated genotypes. The mating data and symmetrical appearance of the phenotypes suggested that a three-allele system (genes β^A, β^D, and β^E) with no dominance is operative. The homozygotes type I (β^A/β^A), type IV (β^D/β^D), and type VI (β^E/β^E) are individually recognizable. The heterozygotes are also recognizable as such; thus type II (β^A/β^D) is clearly a composite of types I and IV, type III (β^D/β^E) a composite of types IV and VI, and type V (β^A/β^E) a composite of types I and IV. Equal mixtures of the appropriate homozygous β-globulin sera are

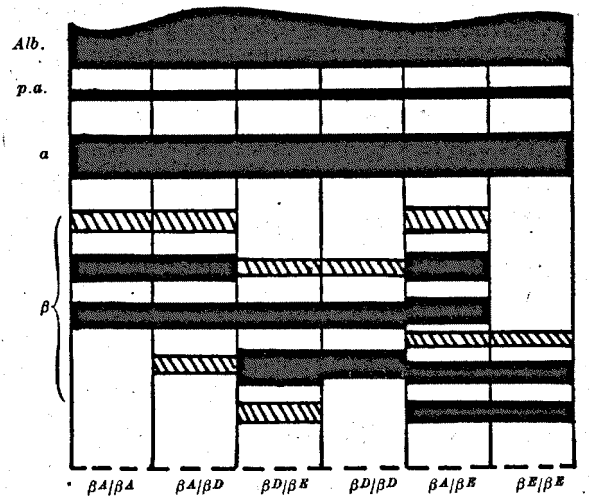


Fig. 1. The six β-globulin types. *Alb.*, albumin; *p.a.*, post albumin; *a*, α-complex; *β*, β-globulins. Solid bands, intense staining; cross-hatched, less intense

indistinguishable qualitatively from genuine samples of heterozygous serum when run side by side. Each of the phenotypes I–VI is thus a single genotype, and it is therefore appropriate to discard the numerical terminology and name each phenotype by its genotype. The expected distribution of phenotypes (genotypes) is shown in Table 1, while pooled mating data for a number of breeds are shown in Table 2. The expected and observed results agree when tested by the χ^2 test.

In the preliminary communication[1] only five phenotypes had been recognized, and to fit the mating data reported at that time it was necessary to

Table 1. Expected Mating Results and Distribution of Offspring on Three-allele Theory

Parents	Distribution of offspring					
	β^{AA}	β^{AD}	β^{DE}	β^{DD}	β^{AE}	β^{EE}
$\beta^{AA} \times \beta^{AA}$	1·0	—	—	—	—	—
,, β^{AD}	0·5	0·5	—	—	—	—
,, β^{DE}	—	0·5	—	—	0·5	—
,, β^{DD}	—	1·0	—	—	—	—
,, β^{AE}	0·5	—	—	—	0·5	—
,, β^{EE}	—	—	—	—	1·0	—
$\beta^{AD} \times \beta^{AD}$	0·25	0·5	—	0·25	—	—
,, β^{DE}	—	0·25	0·25	0·25	0·25	—
,, β^{DD}	—	0·5	—	0·5	—	—
,, β^{AE}	0·25	0·25	0·25	—	0·25	—
,, β^{EE}	—	—	0·5	—	0·5	—
$\beta^{DE} \times \beta^{DE}$	—	—	0·5	0·25	—	0·25
,, β^{DD}	—	—	0·5	0·5	—	—
,, β^{AE}	—	0·25	0·25	—	0·25	0·25
,, β^{EE}	—	—	0·5	—	—	0·5
$\beta^{DD} \times \beta^{DD}$	—	—	—	1·0	—	—
,, β^{AE}	—	0·5	0·5	—	—	—
,, β^{EE}	—	—	1·0	—	—	—
$\beta^{AE} \times \beta^{AE}$	0·25	—	—	—	0·5	0·25
,, β^{EE}	—	—	—	—	0·5	0·5
$\beta^{EE} \times \beta^{EE}$	—	—	—	—	—	1·0

Table 2

Parents	Distribution of 325 single offspring					
	β^{AA}	β^{AD}	β^{DE}	β^{DD}	β^{AE}	β^{EE}
$\beta^{AA} \times \beta^{AA}$	13	—	—	—	—	—
,, β^{AD}	23	30	—	—	—	—
,, β^{DE}	—	3	—	—	4	—
,, β^{DD}	—	42	—	—	—	—
,, β^{AE}	5	—	—	—	3	—
,, β^{EE}	—	—	—	—	2	—
$\beta^{AD} \times \beta^{AD}$	10	14	—	8	—	—
,, β^{DE}	—	1	2	1	3	—
,, β^{DD}	—	32	—	39	—	1
,, β^{AE}	3	3	4	—	1	—
$\beta^{DE} \times \beta^{DE}$	—	—	7	3	—	2
,, β^{DD}	—	—	6	12	—	—
,, β^{AE}	—	1	3	—	1	3
$\beta^{DD} \times \beta^{DD}$	—	—	—	27	—	—
,, β^{AE}	—	4	2	—	—	—
,, β^{EE}	—	—	4	—	—	—
$\beta^{AE} \times \beta^{AE}$	1	—	—	—	2	1

No matings between β^{EE} and β^{AD}, β^{DE}, β^{AE} or β^{EE} have been observed

postulate a series of linked genes. However, with the finding of the sixth phenotype (β^{EE}) the genetic mechanism became clear and it was apparent that five of the 150 matings previously reported[1] (two I × IV → I; one I × IV → IV; one III × V → I; one III × V → IV) were not possible on the 3-allele theory indicated by the sixth phenotype. These mating results were re-investigated and in each case proved to be false after critical examination. In this connexion Rendel[2] found that the stated parentage was in error in about 8 per cent of sire-dam-offspring families blood-grouped in Sweden. It is thus probable that the β-globulin system will be of assistance in checking parentage.

The five phenotypes previously reported[1] were found independently by Smithies and Hickman[3]; on the basis of 71 mating results within two herds they postulated a three-gene theory for cattle β-globulins also, although their interpretation of the expression of each gene has proved to be incorrect.

In order to establish reliable gene frequency data for each breed of cattle it is necessary to examine a representative cross-section of each breed. It is difficult to get a truly random sample by examining cattle indiscriminantly, due to the preponderance of herds using one or two bulls only. However, with the advent of artificial insemination representative groups of pedigree bulls drawn from many sources have been established, and these bulls each serve many hundreds of cows and heifers every year.

Table 3 shows the distribution of phenotypes in bulls standing at Milk Marketing Board cattle-breeding centres compared with bulls standing at other centres.

Gene frequencies for each breed from both groups of centres are shown in Table 4. The results were calculated from the relationships, $A = (2\beta^{AA} + \beta^{AD} + \beta^{AE})/2N$, $D = (\beta^{AD} + \beta^{DE} + 2\beta^{DD})/2N$, and $E = (\beta^{DE} + \beta^{AE} + 2\beta^{EE})/2N$, where β^{AA}, β^{AD}, etc. are the numbers of animals of this phenotype found for any given breed and N is the total number of animals, and where A, D and E are the frequencies of genes β^A, β^D and β^E respectively. The standard errors of the gene frequency estimates were calculated from the expression $\sqrt{1/2N.\ [g(1-g)]}$, where g is gene frequency.

It will be seen that the gene frequencies calculated from both groups of breeding centres are consistent for each breed, within the standard errors found. The gene frequencies found for each breed, therefore, are probably fairly representative for that breed in Britain.

It is evident that the frequency of β^E is greater in

those breeds originating in the climatically more severe parts of the British Isles. Thus β^E is absent in Jersey, Guernsey, Devon and South Devon cattle, confined mainly to the south and south-west of England, while it is most frequent in cattle of Scottish origin. Furthermore, the frequency of this gene within the Ayrshire breed is associated with the location of the herd. In a herd using artificial insemination selected at random in Essex the frequency of β^E was $0{\cdot}118 \pm 0{\cdot}025$ (80 animals), which is in agreement with the expected value (cf. Table 3). In a comparable herd (67 animals) in Aberdeenshire, on the other hand, the frequency of β^E was $0{\cdot}261 \pm 0{\cdot}037$, while Hickman and Smithies[3] found a frequency of $0{\cdot}279 \pm 0{\cdot}049$ in an Ontario Ayrshire herd (42 animals). These results suggest that β-globulin polymorphism in cattle may be concerned with climate

Table 3. DISTRIBUTION OF PHENOTYPES OF 808 PEDIGREE BULLS AT CATTLE-BREEDING CENTRES

Breed	Origin*	Distribution of phenotypes					
		β^{AA}	β^{AD}	β^{DE}	β^{DD}	β^{AE}	β^{EE}
Friesian	*M*	58	85	8	54	9	0
	O	9	16	1	14	7	0
Shorthorn	*M*	42	42	1	18	5	0
	O	16	11	0	6	0	0
Hereford	*M*	16	21	1	27	1	0
	O	3	2	0	6	0	0
Ayrshire	*M*	6	26	14	32	3	0
	O	3	16	3	18	3	0
Aberdeen Angus	*M*	18	7	0	4	9	0
	O	5	4	1	2	2	0
Guernsey	*M*	10	19	—	8	—	—
	O	6	11	—	8	—	—
Jersey	*M*	17	15	—	4	—	—
	O	7	5	—	1	—	—
Devon	*M* and *O*	5	7	—	12	—	—
South Devon	*M* and *O*	1	4	—	9	—	—
Galloway	*M*	0	1	0	0	1	2

* *M*, Milk Marketing Board cattle-breeding centres (22); *O*, other cattle-breeding centres (9).

Table 4. FREQUENCIES OF GENES β^A, β^D AND β^E AND THEIR STANDARD ERRORS

Breed	Origin*	Gene frequencies			Standard errors ±		
		β^A	β^D	β^E	β^A	β^D	β^E
Friesian	*M*	0·490	0·470	0·040	0·024	0·024	0·062
	O	0·436	0·479	0·085	0·051	0·052	0·029
Shorthorn	*M*	0·606	0·366	0·028	0·032	0·033	0·011
	O	0·651	0·349	0·000	0·058	0·058	—
Hereford	*M*	0·409	0·576	0·015	0·043	0·043	0·011
	O	0·364	0·636	0·000	0·102	0·102	—
Ayrshire	*M*	0·253	0·642	0·105	0·041	0·038	0·024
	O	0·291	0·639	0·070	0·049	0·052	0·028
Aberdeen Angus	*M*	0·684	0·198	0·118	0·075	0·065	0·017
	O	0·572	0·321	0·107	0·106	0·100	0·066
Guernsey	*M*	0·527	0·473	—	0·058	0·058	—
	O	0·460	0·540	—	0·071	0·071	—
Jersey	*M*	0·681	0·319	—	0·055	0·055	—
	O	0·792	0·208	—	0·083	0·083	—

* *M* and *O* as in Table 3.

tolerance. It is significant that the average heat adaptability coefficients of some breeds of cattle, quoted by Findlay[4], are in inverse order to the β^E frequencies; for example, Jersey adaptability coefficient 79, β^E nil; Hereford 73, β^E 0·013; Aberdeen Angus 59, β^E 0·115. In view of the high adaptability coefficient (89) of Brahman cattle, it will be worth while investigating the β-globulins of these animals.

It is noteworthy that recently described β-globulin polymorphism in humans sharply distinguishes the black and white races[5]. Polymorphism involving the β-globulins has also been found in sheep[6], goats, horses and rhesus monkeys[7]. This may prove to be one mechanism, therefore, by which a species is able to adapt itself to both temperate and tropical climates.

I gratefully acknowledge the co-operation of the veterinary officers and managers of the various artificial insemination centres in sending samples of bull blood, and of Mr. W. Thomson, of the Duthie Experimental Stock Farm, Aberdeenshire, for supplying blood samples from Ayrshire cattle. I am indebted to Mr. J. C. Gower of Rothamsted for help with the calculation of gene frequencies and errors.

[1] Ashton, G. C., *Nature*, **180**, 917 (1957).
[2] Rendel, J., *Animal Breeding Abs.*, **25**, 223 (1957).
[3] Smithies, O., and Hickman, C. G., *Genetics* (in the press).
[4] Hammond, J., ed., "Progress in the Physiology of Farm Animals" **1**, 264 (Butterworths, London, 1954).
[5] Smithies, O., *Nature*, **180**, 1482 (1957).
[6] Ashton, G. C., *Nature*, **181**, 849 (1958).
[7] Ashton, G. C. (unpublished observations).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

COMMONWEALTH OF AUSTRALIA
COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

(*Reprinted from Nature, Vol.* 184, *pp.* 1135–1136, *Oct.* 10, 1959)

β-GLOBULIN ALLELES IN SOME ZEBU CATTLE

By G. C. ASHTON

National Cattle Breeding Station, Commonwealth Scientific and Industrial Research Organization Laboratory, Rockhampton, Queensland

THREE β-globulin alleles (β^A, β^D and β^E) have been described so far in British breeds of cattle[1]. Two further alleles have now been recognized in zebu (*Bos indicus*) breeds. It is proposed to call these alleles β^B and β^F.

In most mammals the β-globulin alleles give rise to more than one zone by starch-gel electrophoresis[2]. Each of the cattle β-globulin alleles β^A, β^D and β^E gives four detectable zones, the most rapidly migrating zone staining faintly with nigrosine, the next staining moderately and the slowest two zones staining intensely. The two further alleles which have been detected also give rise to four similarly staining zones. The relative mobilities of the groups of four zones controlled by the five alleles are shown in Fig. 1.

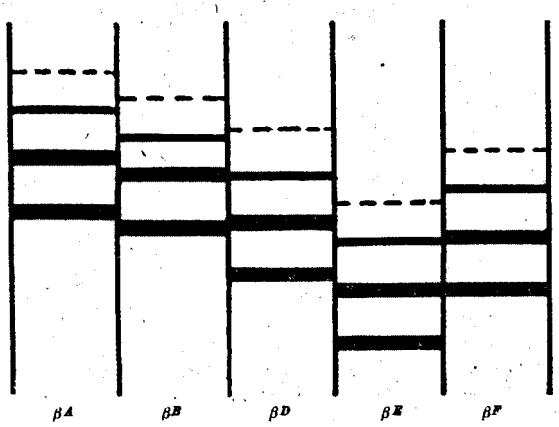


Fig. 1. Relative mobility in starch-gel of the four zones produced by each β-globulin allele in cattle. The anodic side of the gel is at the top of the diagram, only the β-globulin zones being shown

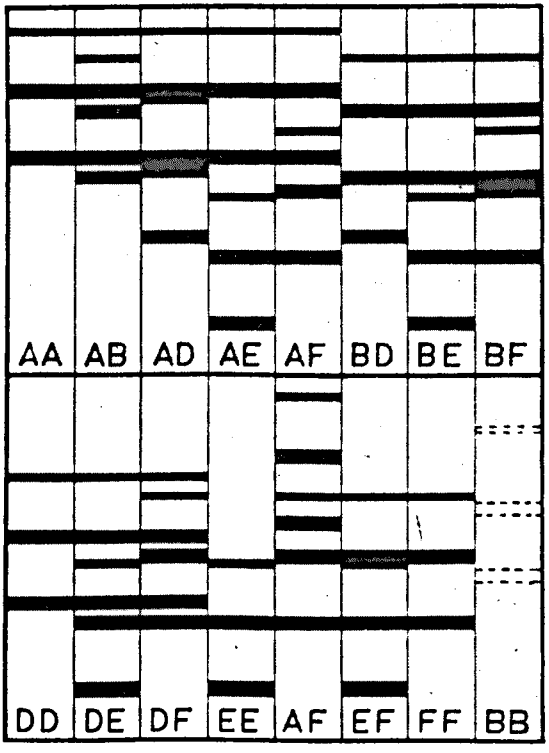


Fig. 2. The fourteen cattle β-globulin phenotypes. The anodic side of the gel is at the top of each portion of the diagram, only the β-globulin zones being shown. The fast-moving faint band produced by each allele (cf. Fig. 1) is not shown. For *AA*, *AB*, etc., read β^{AA}, β^{AB}, etc. The dotted zones for β^{BB} (bottom right) show the expected appearance of this phenotype, which has not yet been found

It will be seen that the zones controlled by β^B are intermediate in mobility to those produced by β^A and β^D, while β^F gives rise to zones intermediate in mobility between those produced by β^D and β^E.

Previous experience has shown that each β-globulin genotype formed from the alleles β^A, β^D and β^E gives only one phenotype[1]. Fifteen phenotypes would therefore be expected from five alleles; fourteen have been found so far. The homozygote of the infrequent allele β^B has not yet been seen. The appearance of the phenotypes (Fig. 2) was anticipated in the main from the knowledge that the pattern given by a heterozygote is indistinguishable from that given by a simple mixture of the corresponding homozygous sera[1,2].

Table 1. DISTRIBUTION OF PHENOTYPES FROM MATINGS INVOLVING THE CATTLE β-GLOBULIN ALLELES β^E AND β^F

Parents		Offspring		
Dam	Sire	Like dam	Like sire	Recombinants
AA^a	AF	3	3	—
AA	BF	—	—	1 AB
AD	AF	1	9	3 AA, 5 DF
AD	BF	—	—	1 AB, 1 DF
AD	FF	—	—	1 DF
AE	AF	5	0	0
BF	AF	1	0	0
DD	AF	—	—	1 AD, 4 DF
DD	DF	0	1	—
EF	AF	0	1	1 FF
EF	DF	1	1	0

* For AA, AF, etc., read βAA, βAF, etc.

The frequency of each allele for several breeds and crossbreeds of cattle from two herds is shown in Table 2.

Table 2. SHOWING β-GLOBULIN GENE FREQUENCIES FOR SOME BREEDS AND CROSS-BREEDS OF CATTLE AT F. D. MCMASTER FIELD STATION (TOP) AND NATIONAL CATTLE BREEDING STATION (BOTTOM)

Breed	No. of animals	Gene frequency				
		βA	βE	βD	βE	βF
Sindhi	14	0·57	0·04	Nil	0·28	0·11
Sindhi × Jersey	29	0·52	Nil	0·24	0·12	0·12
Sahiwal	10	0·10	0·20	Nil	0·15	0·55
Sahiwal × Jersey	31	0·33	0·05	0·28	0·03	0·31
Jersey	51	0·51	Nil	0·49	Nil	Nil
Hereford	27	0·39	Nil	0·52	0·09	Nil
Shorthorn	18	0·56	Nil	0·39	0·05	Nil
Hereford × Shorthorn	10	0·45	Nil	0·55	Nil	Nil
Brahman × Shorthorn	14	0·28	Nil	0·43	0·18	0·11
Brahman × Hereford	15	0·40	Nil	0·24	0·20	0·16
Africander × Shorthorn	13	0·19	Nil	0·50	0·31	Nil
Africander × Hereford	15	0·23	Nil	0·47	0·30	Nil
Brahman*	—	0·3	Nil	0·1	0·3	0·3
Africander*	—	Nil	Nil	0·4	0·6	Nil

* Approximate frequencies computed from remainder of data.

Data from matings between Sindhi × Sindhi, Sahiwal × Sahiwal, Sindhi × Jersey and Sahiwal × Jersey cattle confirm that the previously unrecognized phenotypes represent individual genotypes formed from five alleles (Table 1).

It has been suggested previously[1,3] that the frequency of β^E within a breed may reflect the climatic or ecological stress to which the breed is subjected. Thus, the frequency of β^E increases in a northerly direction in the British Isles, both within

and between breeds. The high frequency of β^E in all the zebu breeds examined is particularly interesting therefore in view of the well-known climatic and ecological tolerance of these cattle.

I thank the Officers-in-Charge of the F. D. McMaster Field Station of the Commonwealth Scientific and Industrial Research Organization, Badgery's Creek, New South Wales, and of the National Cattle Breeding Station of the Commonwealth Scientific and Industrial Research Organization, 'Belmont', Rockhampton, Queensland, for supplying blood samples, and C. Bloomfield for technical assistance.

[1] Ashton, G. C., *Nature*, **182**, 370 (1958).
[2] Ashton, G. C., and McDougall, E. I., *Nature*, **182**, 945 (1958).
[3] Ashton, G. C., *Nature*, **183**, 404 (1959).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Reprinted from the
AUSTRALIAN JOURNAL OF BIOLOGICAL SCIENCES
VOLUME 18, NUMBER 3, PAGES 665–70, JUNE 1965

SERUM TRANSFERRIN *D* ALLELES IN AUSTRALIAN CATTLE

By G. C. Ashton

Reprinted for the
Commonwealth Scientific and Industrial Research Organization
Australia

SERUM TRANSFERRIN *D* ALLELES IN AUSTRALIAN CATTLE

By G. C. Ashton*

[*Manuscript received November* 30, 1964]

Summary

Transferrin phenotypes supporting the occurrence of two Tf^D alleles in cattle are illustrated. Segregation data and gene frequencies for five breeds for the two alleles Tf^{D1} and Tf^{D2} are presented.

I. Introduction

Serum transferrin polymorphism in *Bos taurus* cattle was described independently by Ashton (1957, 1958) and by Hickman and Smithies (1957) and Smithies and Hickman (1958). Each laboratory presented evidence for control by three co-dominant autosomal alleles which are now known as Tf^A, Tf^D, and Tf^E. Subsequently Ashton (1959) reported two further alleles, Tf^B and Tf^F, found so far only in *B. indicus* cattle, and Ashton and Lampkin (1964) reported another allele, Tf^G, found so far only in three related Boran cattle in East Africa. Gahne (1961) has described a phenotype, found in Icelandic cattle, which may represent the heterozygote of Tf^A and yet another allele similar in mobility to Tf^F.

Each of these transferrin alleles produces four zones in starch gel (Ashton and McDougall 1958), although in routine analysis the fastest zone is rather faint and is not always seen. This characteristic of three major zones per allele permits prediction of the appearance of the whole range of possible phenotypes when a "new" allele is discovered (Ashton 1959).

In 1962 Kristjansson reported that cattle Tf^D is in fact two alleles, each of which produces zones of very similar mobility in starch gel. A similar situation has arisen with sheep transferrins (Ashton and Ferguson 1963). The resolution of zones of very similar mobility has been accomplished by development of discontinuous buffer systems based on the original description of such systems by Poulik (1959).

The purpose of this paper is to illustrate the relationship of the two *D* alleles to the other transferrin alleles in *B. taurus* and *B. indicus* cattle and to present data on their distribution in some Australian breeds. The two *D* alleles have been coded Tf^{D1} and Tf^{D2}; Tf^{D1} producing zones of slightly faster mobility than Tf^{D2}. Samples were exchanged with Dr. F. K. Kristjansson in Ottawa, and it was found that the two alleles coded Tf^{D1} and Tf^{D2} in Rockhampton corresponded with the two alleles coded Tf^D and Tf^{D1} by Kristjansson (1962). It has been agreed to code these alleles Tf^{D1} and Tf^{D2} (Ashton and Kristjansson 1965).

* Cattle Research Laboratory, Division of Animal Genetics, CSIRO, Rockhampton, Qld.; present address: Department of Genetics, University of Hawaii, Honolulu, Hawaii.

II. Methods

(a) *Starch-gel Electrophoresis*

The system used has been described previously (Ashton and Braden 1961; Ashton and Ferguson 1963; Ashton and Lampkin 1965) and has been in use since 1959. It is based on the discontinuous buffer system of Poulik (1959), and was developed by Dr. K. A. Ferguson for resolution of pituitary proteins in starch gel. It differs from Poulik's system firstly in the use of lithium hydroxide instead of sodium hydroxide, which lowers conductivity and hence lessens heat production without sacrificing ionic strength, and secondly in the admixture of a proportion of electrolyte with the gel buffer. This has the effect of slowing the transferrin zones relative to albumin, and gives excellent resolution of the numerous zones between the trailing edge of the albumin zone and the leading transferrin zone.

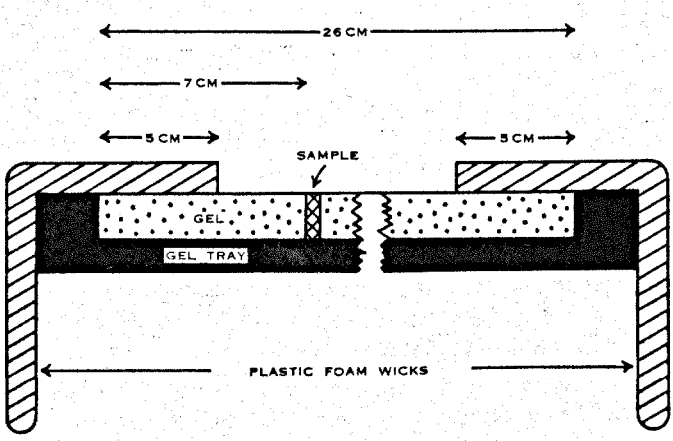


Fig. 1.—Showing gel dimensions and position of insertion of sample (on Whatman 17 filter paper) in relation to wicks.

The electrolyte consists of 0·75 g lithium hydroxide and 11·8 g boric acid per litre of solution, giving a pH of 7·8. The gel buffer consists of 450 ml of a solution containing 1·6 g citric acid and 4·8 g tris(hydroxymethyl)aminomethane per litre (pH 8·0) and 50 ml of electrolyte. Gels are prepared from Connaught hydrolysed starch (Connaught Laboratories, Toronto, Canada) at the concentration recommended by the manufacturer, by the method described by Kristjansson (1963). The gels are cast in moulds 26 cm long divided into units 4 cm wide and 0·3 cm deep. The gels are used within 2 hr of pouring.

The dimensions of the gel are fairly critical especially in regard to the point of sample insertion. Figure 1 shows the relationship of connecting wicks, sample insertion, and mould length. Samples are inserted on pieces of Whatman 17 filter paper, and removed after 20 min electrophoresis. During electrophoresis the gels are covered with thin polyvinyl film to prevent evaporation.

The applied voltage should be sufficiently high to effect rapid migration, but not so high that the gels get too hot. Gel temperatures of 40–45°C do not hinder resolution, and in fact seem to enhance it. With an applied voltage of 400 V across the apparatus, and an initial current of 4 mA per centimetre width of gel, electrophoresis is complete in 2–2½ hr. The voltage drop between the inside edges of the wicks is 180–190 V. Electrophoresis is terminated when the brown zone of discontinuity just reaches the inside edge of the anode wick.

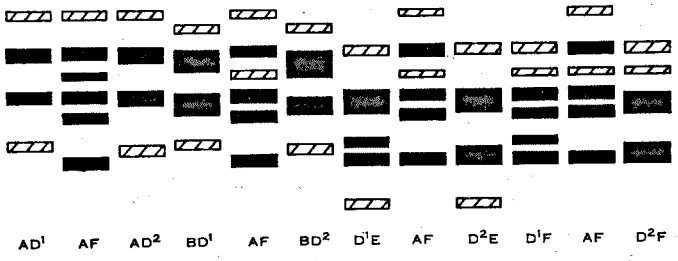


Fig. 2.—Showing pairs of *D* phenotypes referred to transferrin *AF*. The faint fastest zone produced by each allele is not shown. The cross-hatched areas indicate zones less intensely stained than the solid areas. See also Plate 1.

The undersurface of the gel is stained, either in 0·05% nigrosine or 0·1% naphthalene black in methanol–water–acetic acid (50 : 50 : 10 by vol.) and the gel is not sliced.

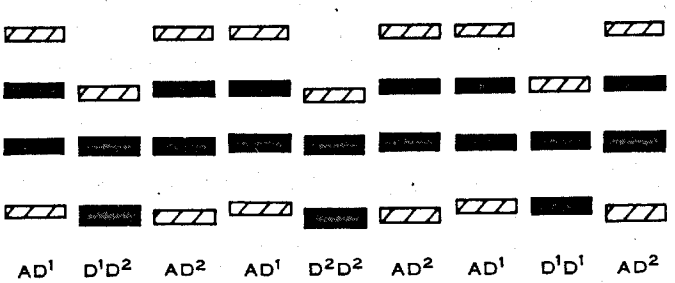


Fig. 3.—Showing *D* phenotypes referred to transferrin phenotypes D^1D^1, D^1D^2, and D^2D^2. The faint fastest zone produced by each allele is not shown. The cross-hatched areas indicate zones less intensely stained than the solid areas. See also Plate 1.

III. Results

(*a*) *Identification of Phenotypes*

Plate 1 shows some of the 21 phenotypes possible with the six alleles Tf^A, Tf^B, Tf^{D^1}, Tf^{D^2}, Tf^E, and Tf^F.

Distinction between Tf^{D^1} and Tf^{D^2} is most easily made in the heterozygous transferrin pairs AD^1 and AD^2, BD^1 and BD^2, D^1E and D^2E, D^1F and D^2F (Plate 1). Figure 2 illustrates the differences diagrammatically, in each case against transferrin AF which has been found most useful as a general reference for all cattle transferrin phenotypes except as noted below.

TABLE 1

RESULTS OF 71 MATINGS INVOLVING D PHENOTYPES

Parents	Phenotypes of Offspring	Parents	Phenotypes of Offspring
$AA \times D^1D^2$	1 AD^2	$EE \times D^1D^2$	1 D^2E
$AA \times D^2D^2$	3 AD^2	$EE \times D^2D^2$	3 D^2E
$AA \times AD^1$	4 AD^1	$EE \times AD^1$	1 AE
$AA \times AD^2$	13 AA; 11 AD^2	$EE \times AD^2$	4 AE; 5 D^2E
$AA \times D^2E$	1 AE	$EE \times D^2E$	1 D^2E
$AF \times D^1D^2$	1 AD^2; 1 D^2F	$FF \times D^1D^2$	4 D^2F
$AF \times D^2D^2$	1 AD^2	$FF \times AD^1$	1 AF
$AF \times AD^1$	2 AA; 1 AF; 1 D^1F	$FF \times AD^2$	6 AF; 5 D^2F
$AF \times AD^2$	3 AA; 2 AF; 3 D^2F; 3 AD^2		

The most difficult phenotypes to distinguish are D^1D^1, D^1D^2, and D^2D^2. Logically a D^1D^2 reference sample could be used, but in practice either AD^1 or AD^2 has proved more reliable (see Plate 1 and Fig. 3).

TABLE 2

GENE FREQUENCIES CALCULATED FROM PHENOTYPE DISTRIBUTIONS IN AUSTRALIAN COW AND BULL POPULATIONS

Breed	Source	No. of Animals	Gene Frequencies					
			Tf^A	Tf^B	Tf^{D^1}	Tf^{D^2}	Tf^F	Tf^E
Jersey	Cows in six herds	578	0·722	—	0·049	0·229	—	—
Jersey	Bulls at artificial breeding centres	51	0·598	—	0·147	0·255	—	—
Guernsey	Cows in one herd	47	0·500	—	0·330	0·170	—	—
Australian Illawarra Shorthorn	Cows in one herd	70	0·343	—	0·143	0·343	—	0·171
Friesian	Bulls at artificial breeding centres	30	0·483	—	0·100	0·367	—	0·050
Droughtmaster	Cows in one herd	293	0·266	0·072	0·041	0·319	0·111	0·191

(*b*) *Segregation of* Tf^{D^1} *and* Tf^{D^2}

Table 1 shows mating data supporting the conclusion that Tf^{D^1} and Tf^{D^2} are allelic to the other transferrin alleles. Because of the relatively low frequency of Tf^{D^1} in Australian cattle so far examined the critical matings $D^1D^2 \times D^1D^2$ and $D^1D^1 \times D^1D^1$

have not yet been encountered. No examples of a mating involving only the Tf^{D1} allele (in combination with other non-*D* alleles) giving a Tf^{D2} offspring have occurred. Similarly no matings involving the Tf^{D2} allele only have given progeny with Tf^{D1}.

(c) *Breed Distribution of* Tf^{D1} *and* Tf^{D2}

Table 2 shows the distribution of Tf^{D1} and Tf^{D2} and the calculated gene frequencies for Jersey, Guernsey, Australian Illawarra Shorthorn, and Droughtmaster breeds in Australia. These represent a limited number of herds and must be considered as preliminary estimates of breed gene frequencies. However, it would appear that Tf^{D2} is more frequent than Tf^{D1}, except in Guernsey cattle.

IV. Discussion

Recognition of "new" alleles presents several problems of which nomenclature is not the least. If the new allele produces zones clearly distinguishable from those of existing alleles, as in the case of Tf^{B}, Tf^{F}, and Tf^{G}, the practice of assigning the next available letter has some merit. However, it is essential that the same letter should not be allotted to different newly observed alleles. The establishment of a subcommittee to consider nomenclature of serum protein polymorphisms in farm livestock (cf. 1st Report of FAO Panel of Blood Group Scientists, AN 1963/7, Rome) should be useful in avoiding this pitfall. The problem of coding alleles which are recognized by improvement in technique, as in the case of the transferrin *D* alleles, is more complex. Coding each of the "new" alleles by addition of superscript numbers is unambiguous. It has the advantage of showing relationship to the previous coding, while emphasizing that the "new" alleles do not correspond with the old.

Recognition of two *D* alleles means that data relating transferrin type to milk yield (Ashton 1960; Ashton, Fallon, and Sutherland 1964) and transferrin type to fertility (Ashton and Fallon 1962) need reassessment. Preliminary results show that in the case of fertility at least there is no significant difference between D^1D^1, D^2D^2, and D^1D^2 bulls. The ranking of D^1D^1, D^2D^2, and D^1D^2 cows with regard to milk yield is being investigated.

V. Acknowledgments

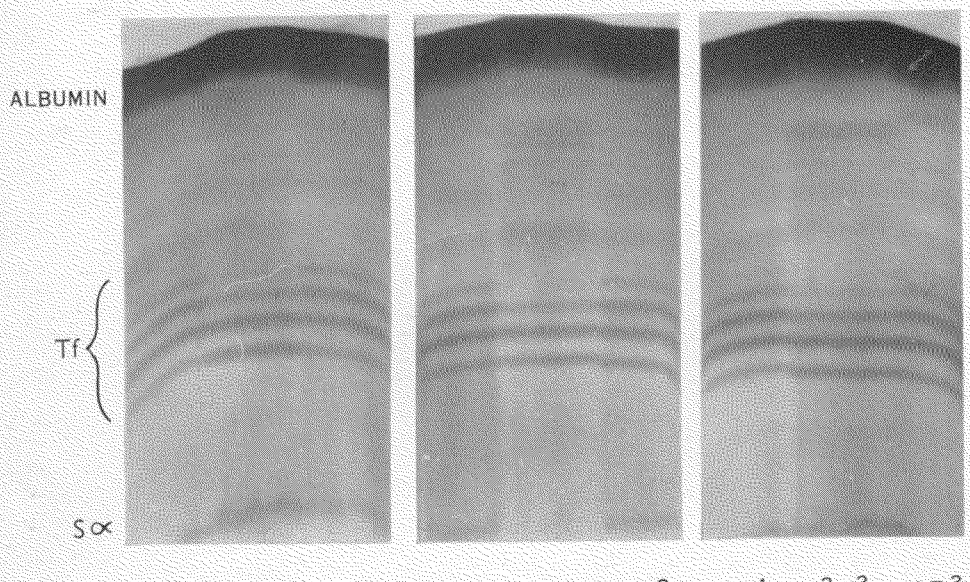
Mr. M. N. Dennis and Miss M. Greenwood gave capable technical assistance.

VI. References

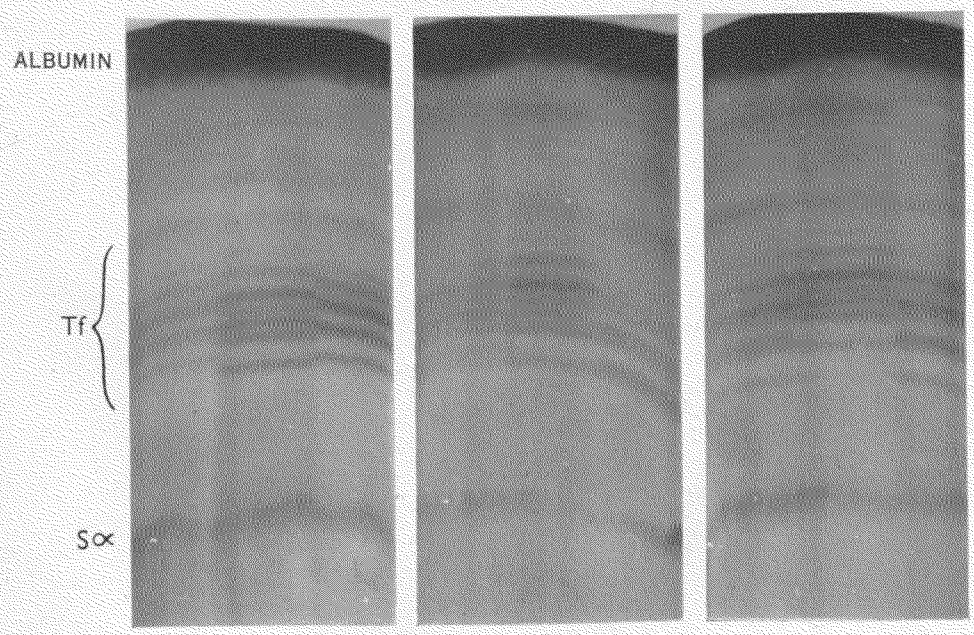
Ashton, G. C. (1957).—*Nature, Lond.* **180**: 917.
Ashton, G. C. (1958).—*Nature, Lond.* **182**: 370.
Ashton, G. C. (1959).—*Nature, Lond.* **184**: 1135.
Ashton, G. C. (1960).—*J. Agric. Sci.* **54**: 321.
Ashton, G. C., and Braden, A. W. H. (1961).—*Aust. J. Biol. Sci.* **14**: 248.
Ashton, G. C., and Fallon, G. R. (1962).—*J. Reprod. Fert.* **3**: 93.
Ashton, G. C., Fallon, G. R., and Sutherland, D. N. (1964).—*J. Agric. Sci.* **62**: 27.
Ashton, G. C., and Ferguson, K. A. (1963).—*Genet. Res.* **4**: 240.
Ashton, G. C., and Kristjansson, F. K. (1965).—*Immunogenet. Lett.* **4**(2): (in press).
Ashton, G. C., and Lampkin, G. H. (1965).—*Nature, Lond.* **205**: 209.
Ashton, G. C., and McDougall, E. I. (1958).—*Nature, Lond.* **182**: 945.

GAHNE, B. (1961).—*Anim. Prod.* **3**: 135.
HICKMAN, C. G., and SMITHIES, O. (1957).—*Proc. Genet. Soc. Can.* **2**: 39.
KRISTJANSSON, F. K. (1962).—Proc. 8th Annual Blood Group Conference, Ljubljana, Yugoslavia. (Mimeo).
KRISTJANSSON, F. K. (1963).—*Genetics* **48**: 1059.
POULIK, M. D. (1957).—*Nature, Lond.* **180**: 147.
SMITHIES, O., and HICKMAN, C. G. (1958).—*Genetics* **43**: 374.

CATTLE *D* ALLELES



Portion of gels between the Sα (slow α-globulin) zone and the albumin zone showing the five transferrin phenotypes AD^1, AD^2, D^1D^1, D^1D^2, and D^2D^2.



Portion of gels between the Sα (slow α-globulin) zone and the albumin zone showing pairs of *D* alleles against an *AF* reference serum.

(*Reprinted from Nature, Vol.* 183, *pp.* 404–405, *Feb.* 7, 1959)

β-Globulin Polymorphism and Early Fœtal Mortality in Cattle

Six β-globulin types have been found in British breeds of cattle by starch-gel electrophoresis[1]. Mating data showed that the six types represent the individual homozygotes and heterozygotes of three β-globulin alleles, β^A, β^D and β^E. Five of these six types were seen independently by Hickman and Smithies[2], who postulated a similar genetic mechanism.

It has been found that the proportion of the two possible types of offspring from some reciprocal matings is apparently influenced by the maternal genotype. Thus, from certain matings involving only the alleles β^A and β^D equal numbers of offspring like or unlike the mother (with respect to β-globulin genotype) would be expected. However, a consistent excess of offspring like the mother was obtained from such matings in both Friesian and Ayrshire cattle (Table 1). Although several of the calves in the results were sired by the same bull the effect was not correlated with particular bulls. Because of the

Table 1. Reciprocal Matings showing an Excess of Like-Mother Offspring in Two Breeds of Cattle

Mating	Offspring (female calves from cows mated with A.I. bulls)			
	Friesian		Ayrshire	
Dam Sire	Like dam	Like sire	Like dam	Like sire
$\beta^A\beta^A \times \beta^A\beta^D$	19	11	0	0
$\beta^A\beta^D \times \beta^A\beta^A$	13	8	12	3
$\beta^D\beta^D \times \beta^A\beta^D$	17	9	3	1
$\beta^A\beta^D \times \beta^D\beta^D$	32	25	1	0
Totals	81	53	16	4
Significance	$\chi^2 = 5{\cdot}85$; $P = 0{\cdot}01$–$0{\cdot}02$		$\chi^2 = 7{\cdot}2$; $P = 0{\cdot}01$–$0{\cdot}001$	

Table 2. Reciprocal Matings involving the Allele β^E (Results from Several Breeds Pooled)

Mating	Offspring (female calves from cows mated with A.I. bulls)	
Dam Sire	Without β^E	With β^E
$\beta^A\beta^A \times \beta^A\beta^E$ $\beta^D\beta^D \times \beta^D\beta^E$	Like dam 6 22	Like sire 5 11
$\beta^A\beta^E \times \beta^A\beta^A$ $\beta^D\beta^E \times \beta^D\beta^D$	Like sire 5 8	Like dam 1 2

practice of early disposal of bull calves from dairy herds the data relate only to female calves. It is not yet known if the same phenomenon occurs with male offspring.

In similar matings involving the allele β^E a preponderance of like-mother offspring was found when this allele was in the fathers' genotype only (Table 2). From the corresponding reciprocal matings where the mothers' genotype included β^E a consistent lack of β^E offspring was found.

It is unlikely that these effects can be explained by differential production of gametes, by selective fertilization, by differential mortality in calfhood or by unwitting selection by the dairy farmer. The only simple explanation appears to be that the chances of survival of the bovine embryo are affected by the β-globulin genotype of the mother. Thus embryos differing in genotype from the mother appear to be less compatible with the mother than those of like type, except where the maternal genotype includes β^E. Then there seems to be antagonism between β^E dam and β^E embryo causing a dearth of β^E offspring. In this connexion it is significant that β^E is the least frequent of the three alleles in each breed examined, and is in fact absent in Jersey, Guernsey and South Devon cattle. A gene at a disadvantage in a balanced polymorphic system must have some counterbalancing advantage or it would be eliminated by natural selection. Whatever the nature of this advantage for β^E it is presumably greater for northern breeds of British dairy cattle than for southern because the β^E frequency increases steadily northwards[1].

Because of the low frequency of β^E the losses due to the postulated antagonism between β^E dam and embryo would be numerically small. However, the losses due to the lack of unlike-mother offspring (Table 1) from matings involving β^A and β^D could be quite important. Assuming that one-third fewer unlike-mother offspring are produced, it can be calculated that when the frequencies of β^A and β^D are 0·5 and β^E nil, only 83 per cent of conceptions would result in viable offspring if there were no other causes of prenatal loss. This percentage will increase as the disproportion between the frequency of β^A and β^D increases.

Robinson has used data from several sources to compute the estimated extent of prenatal loss in the cow[3]. He concludes that 10 per cent of services to cows in œstrus do not result in fertilization, while the products of conception from 27 per cent die during the first three months of gestation. Data published by the Milk Marketing Board[4] indicate a

consistent loss of about 12 per cent of fœtuses between the first and third months of pregnancy. The loss was unaffected by season for three years, although the overall conception-rate showed seasonal variation. This 12 per cent loss is in close agreement with the estimated average of 86–88 per cent viability due to mother – fœtus β-globulin incompatibility.

From these results one would expect a greater percentage of viable offspring from crosses between homozygous bulls and cows of the same genotype, β^E individuals excluded, than from other crosses. Data to check this are now being collected.

I am indebted to Drs. P. M. Sheppard and A. W. H. Braden for helpful suggestions and criticism.

G. C. Ashton*

Animal Health Trust,
Farm Livestock Research Centre,
Stock,
Essex.

* Present address: C.S.I.R.O. Sheep Biology Laboratory, Prospect, N.S.W., Australia.

[1] Ashton, G. C., *Nature*, **182**, 370 (1958).
[2] Hickman, C. G., and Smithies, O., *Proc. Gen. Soc. Canada*, **2**, 39 (1957).
[3] Robinson, T. J., in "Progress in the Physiology of Farm Animals", edit. by Hammond, J., **3**, 793 (Butterworths, London, 1956).
[4] Production Division Report No. 3, 23 (Milk Marketing Board, Thames Ditton, Surrey, 1952).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Journal of Reproduction and Fertility (1961) **2**, 117-129

β-GLOBULIN TYPE AND FERTILITY IN ARTIFICIALLY BRED DAIRY CATTLE

G. C. ASHTON

National Cattle Breeding Station, C.S.I.R.O., Rockhampton, Queensland, Australia

(*Received* 22*nd September* 1960)

Summary. The serum β-globulin types of 360 cows in fifteen herds of nominally Jersey cattle in the Nambour region of Queensland and 423 cows from nineteen herds of nominally Australian Illawarra Shorthorn cattle in the Kingaroy region were determined, together with the β-globulin types of eighteen Jersey bulls used for artificial insemination in the Nambour region and nine Australian Illawarra Shorthorn bulls used in the Kingaroy region. The results, expressed as breeding efficiencies, of 1527 inseminations from the Jersey bulls and 1166 inseminations from the Australian Illawarra Shorthorn bulls were then examined with respect to the β-globulin types of the bull and cow. It was found that β-globulin type had a highly significant effect on fertility in both regions. In the Nambour region, the breeding efficiency for matings between partners both homozygous at the β-globulin locus was 57·98% compared with 47·68% ($\chi^2 = 11{\cdot}28$, $P<0{\cdot}001$) for partners one or both of which were heterozygous. In the Kingaroy region, the comparable breeding efficiencies were 62·93% and 47·24% ($\chi^2 = 12{\cdot}83$, $P<0{\cdot}001$). In each region, the breeding efficiency with the homozygous bulls was about 4% greater than the mean for the region. The practical significance of these observations is discussed.

INTRODUCTION

During a survey of the serum β-globulins of British dairy cattle (Ashton, 1958), it was found that the proportion of the two possible types of offspring from some reciprocal crosses was apparently influenced by the maternal genotype (Ashton, 1959). From the matings $\beta^{AA}\sigma \times \beta^{AD}\varphi$, $\beta^{AD}\sigma \times \beta^{AA}\varphi$, $\beta^{DD}\sigma \times \beta^{AD}\varphi$ and $\beta^{AD}\sigma \times \beta^{DD}\varphi$, there were, among the female offspring, ninety-seven of the same genotype as the dams and fifty-seven like the sires, a highly significant difference ($\chi^2_1 = 10{\cdot}4$, $P<0{\cdot}01$). Asymmetrical segregation ratios were also found in matings involving the allele β^E, the least frequent of the three alleles present in British breeds of cattle, the results from these matings showing an excess of offspring lacking β^E over offspring with β^E.

From these results, it was suggested that the chances of survival of a bovine embryo are affected by the β-globulin type of its mother and that the fertility of the different β-globulin mating groups might not be the same. This report

describes the results of an investigation into the effect of the β-globulin types of the parents on fertility with data from artificial breeding sub-centres in Queensland. The results confirm that parental β-globulin type does affect fertility, but apparently not in the manner previously postulated.

MATERIALS AND METHODS

MATERIALS

Blood-serum samples were obtained from cows that had been inseminated and from the bulls providing the semen. The β-globulin type was determined. Samples were collected from two localities in Queensland, Nambour and Kingaroy.

DETERMINATION OF β-GLOBULIN TYPE

An individual animal's β-globulin type was determined by starch-gel electrophoresis by a previously described technique (Ashton, 1957, 1960). The method was a slight modification of the original procedure of Smithies (1955) with a different buffer system. It was designed to allow processing of large numbers of samples rapidly and unequivocally for β-globulin type rather than achieving complete resolution of all the serum-protein components present in the sample.

PLAN OF BREEDING PROGRAMME

The general plan of the artificial-breeding programme in Queensland has been described by Fallon (1958). It is essentially a bull-proving project in which a limited number of bulls, held at the Animal Husbandry Research Farm, Rocklea, Brisbane, are tested each year during an artificial breeding season of about 4 months duration. Two sub-centres have been established in southern Queensland for distributing semen from these bulls to dairy farms, one at Nambour and one at Kingaroy.

Nambour region. Three hundred and sixty cows, from fifteen out of about fifty herds in the scheme in this region, were sampled. In these herds, eighteen bulls provided 1527 inseminations through the Nambour sub-centre. All the bulls were pedigree Jersey bulls, and all the cows and heifers inseminated were nominally Jersey. Four bulls were used in 1955, of which three (all β^{AD}) were available for bleeding. Four different bulls were used in 1956, of which three were available for bleeding (two β^{AA} and one β^{AD}). Four different bulls were used in each of the years 1957-59 and all twelve bulls were available for bleeding. Their phenotypes were as follows: in 1957 two β^{AA}, one β^{AD} and one β^{DD}; 1958, three β^{AA} and one β^{AD}; and 1959, two β^{AA} and two β^{AD}.

Kingaroy region. Four hundred and twenty-three cows, from nineteen out of about fifty herds in the scheme in this region, were sampled. In these herds, nine bulls provided 1166 inseminations through the Kingaroy sub-centre. All the bulls were pedigree Australian Illawarra Shorthorn (AIS) bulls, and the cows and heifers inseminated were nominally of the same breed. Five bulls were used in 1958 of which four were available for bleeding. Of these, one was β^{AD}, one β^{DD} and two β^{DE}. In 1959, four different bulls were used, three β^{AD} and one β^{DD}.

ARTIFICIAL BREEDING DATA

The data presented below relate to the artificial insemination of a number of cows over one or more years. The same cow may appear in the data in more than one year. In practice, a given cow is offered by the farmer for insemination when signs of oestrus are detected by him. If oestrus does not reappear within about 5 months after insemination, the insemination is considered successful and a live or stillborn calf usually results. Occasionally, the foetus is aborted. Either eventuality is classed as a 'pregnancy' in the treatment of the data. Alternatively, if the insemination is not successful, oestrus will usually reappear and the cow is said to 'return to service'. The time of reappearance in days after insemination is noted. In the treatment of the data, the returns are grouped into three periodicity classes, short (up to 17 days), normal (18 to 24 days) and long (25 days onwards, until the return follows abortion). These subdivisions are discussed in more detail below. In some cows, there is a return to the anoestrous state following an unsuccessful insemination. In this case the time of return will not be recorded (see footnote, Table 7). In the event of a return, a further insemination is carried out which may or may not be by the same bull. The outcome of the repeat insemination is recorded as a separate result. Up to five inseminations were recorded on some cows in a given year but this was rare.

To avoid confusion, the term 'conception rate', which is widely used in commercial practice and in more than one sense, is not used here. Instead 'breeding efficiency', defined as the percentage of individual inseminations resulting in a live or stillborn calf or an aborted foetus, is used. A 'breeding result' is the outcome of a single insemination, either pregnancy as defined or a return to service. A 'mating group' refers to the β-globulin-phenotype combination of bull and cow; for example, inseminations from β^{AA} bulls into β^{AD} cows would give breeding results falling into the $\beta^{AA}\sigma \times \beta^{AD}\varphi$ mating group.

RESULTS

NAMBOUR BREEDING EFFICIENCIES

Breeding results for 1527 artificial inseminations from eighteen Jersey bulls in the Nambour region over the years 1955-59 are shown in Table 1. For each of the nine possible β-globulin mating groups, the total number of inseminations for any year is shown, together with the breakdown into the number of inseminations causing pregnancy and the number resulting in returns to service. It will be seen that the data are incomplete because there were no β^{AA} bulls in use in 1955, and no β^{DD} bulls in 1955, 1956, 1958 and 1959. Also, because β^{E} is absent in Jersey cattle, there are no matings involving this allele.

The breeding results for the different years have been compared within each mating group (Table 2). There is no evidence of a significant difference between years in breeding results within any mating group, with the exception of the $\beta^{AD}\sigma \times \beta^{DD}\varphi$ group. In this group, the χ^2 value of 13·57 is highly significant. The main cause of the heterogeneity is the data obtained in 1957, which contributed 7·20 to the χ^2 value. Excluding these few results would give a

Table 1

SHOWING THE RESULTS OF 1527 INSEMINATIONS FROM EIGHTEEN JERSEY BULLS DURING FIVE BREEDING SEASONS IN THE NAMBOUR AREA OF QUEENSLAND WITH RESPECT TO β-GLOBULIN TYPE OF COWS AND BULLS

Female phenotype	*Year*	*Male phenotype*											
		βAA				*βAD*				*βDD*			
		*Preg.**	*Ret'd†*	*Total*	%	*Preg.*	*Ret'd*	*Total*	%	*Preg.*	*Ret'd*	*Total*	%
βAA	1955	–	–	–		21	19	40		–	–	–	
	1956	23	15	38		9	10	19		–	–	–	
	1957	26	22	48		14	17	31		12	4	16	
	1958	60	48	108		5	12	17		–	–	–	
	1959	25	23	48		20	44	64		–	–	–	
	Total	134	108	242	55·4	69	102	171	40·4	12	4	16	75·0
βAD	1955	–	–	–		34	43	77		–	–	–	
	1956	38	26	64		21	17	38		–	–	–	
	1957	45	53	98		17	28	45		19	15	34	
	1958	91	86	177		29	28	57		–	–	–	
	1959	48	45	93		40	58	98		–	–	–	
	Total	222	210	432	51·4	141	174	315	44·8	19	15	34	55·9
βDD	1955	–	–	–		20	20	40		–	–	–	
	1956	16	7	23		7	2	9		–	–	–	
	1957	25	18	43		4	16	20		12	10	22	
	1958	35	24	59		11	3	14		–	–	–	
	1959	24	16	40		23	24	47		–	–	–	
	Total	100	65	165	60·6	65	65	130	50·0	12	10	22	54·6

* Pregnant.
† Returned to service.

value of 6·37 which is not significant. The breeding results for the various seasons within a mating group have been pooled, therefore, in further analysis of the data. The aberrant 1957 $\beta^{AD}\sigma^{\prime} \times \beta^{DD}\varphi$ results have been retained as they have little influence on the subsequent comparisons.

TABLE 2

SIGNIFICANCE OF DIFFERENCES IN BREEDING EFFICIENCY BETWEEN YEARS WITHIN SIX OF THE NINE POSSIBLE β-GLOBULIN MATING GROUPS IN THE NAMBOUR DATA

Female phenotype	*Male phenotype*			
	βAA		*βAD*	
	χ^2§	*P*	χ^2†	*P*
βAA	0·827	>0·80	6·18	>0·10
βAD	2·806	>0·50	4·07	>0·50
βDD	0·886	>0·80	13·57	<0·01

Matings involving β^{DD} bulls occurred in one year only.
§ Three degrees of freedom.
† Four degrees of freedom.

Pooling the breeding results for the different years gives a single result for each of the nine possible mating groups. Comparison of these pooled breeding results with the mean breeding efficiency for all the Nambour inseminations shows that some of them differ significantly from the mean. The pooled data are, in fact, very significantly heterogeneous ($\chi^2_8 = 24{\cdot}76$, $P<0{\cdot}01$). Because the differences are not due to variation within the breeding groups as a consequence of pooling data for several years, it can be concluded that there are real differences in breeding efficiency between the β-globulin mating groups.

Examination of the pooled results suggested that the nine mating groups could be rearranged into four combined mating groups on the basis of both breeding efficiencies and genetic association. There is no evidence of a significant difference in breeding efficiency between the mating groups which have been combined. The four combined mating groups have been composed as follows (bulls' phenotype first):

1. Unlike homozygotes, $\beta^{AA} \times \beta^{DD}$ and $\beta^{DD} \times \beta^{AA}$.
   Mean breeding efficiency 61·9%.
2. Like homozygotes, $\beta^{AA} \times \beta^{AA}$ and $\beta^{DD} \times \beta^{DD}$.
   Mean breeding efficiency 55·3%.
3. Homozygote × heterozygotes, $\beta^{AA} \times \beta^{AD}$, $\beta^{AD} \times \beta^{AA}$, $\beta^{DD} \times \beta^{AD}$ and $\beta^{AD} \times \beta^{DD}$.
   Mean breeding efficiency 48·6%.
4. Heterozygote × heterozygotes, $\beta^{AD} \times \beta^{AD}$.
   Mean breeding efficiency 45·0%.

Comparison of the combined-mating-groups breeding efficiencies has been made by a modification of the χ^2 test suggested by Woolf (1955) for estimating

the relationship between blood group and disease. This consists of comparing the ratios of non-returns and returns for the two mating groups for corresponding years, and calculating the magnitude of the discrepancy both between years and overall. The χ^2 value for discrepancy (heterogeneity) in the data between years is subtracted from the overall χ^2 value to give a corrected value for discrepancy between mating-group breeding efficiencies. The comparisons between the combined mating groups are shown in Table 3. None of the heterogeneity χ^2 values are significant. It is evident that like-and-unlike-homozygote breeding results are not significantly different, nor are homozygote × heterozygote and heterozygote × heteroyzgote results. However, unlike-homozygote and homozygote × heterozygote breeding results are very significantly different and so are unlike-homozygote and heterozygote × heterozygote results. Like-homozygote matings differ significantly from heterozygote × heterozygote breeding results, but not from homozygote × heterozygote results.

TABLE 3

SIGNIFICANCE OF THE COMPARISON BETWEEN THE NAMBOUR COMBINED MATING GROUPS MEAN BREEDING EFFICIENCIES BY WOOLF'S (1955) METHOD

Comparison	*Like homozygotes*	*Homozygote × heterozygotes*	*Heterozygote × heterozygotes*
Unlike homozygotes	(0·236) 1·974	(0·989) 9·790**	(1·569) 10·902***
Like homozygotes	–	(0·563) 2·643	(1·068) 4·082*
Homozygote × heterozygotes	–	–	(0·401) 0·897

The χ^2 values shown are corrected for heterogeneity due to years. The heterogeneity χ^2 values are shown in brackets, none of the values being significant (three degrees of freedom).

* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0·1% level.

It seems reasonable to go further, therefore, and pool all matings between homozygotes (mean breeding efficiency 58·0%), on the one hand, and all matings in which heterozygotes are involved (mean breeding efficiency 47·7%), on the other. The comparison shows that matings between homozygotes are very significantly superior (corrected $\chi^2 = 11{\cdot}28$; $P<0{\cdot}01$).

KINGAROY BREEDING EFFICIENCIES

Breeding results for 1166 artificial inseminations from nine AIS bulls in the Kingaroy region in 1958 and 1959 are shown in Table 4. Three alleles, β^A, β^D and β^E are present in AIS cattle and thirty-six mating groups are possible. However, no β^{AE} or β^{EE} bulls were used in this region, and data are available for twenty-four mating groups only. Very few β^{EE} cows were sampled and only seventeen matings between β^{EE} cows and the bulls used were recorded.

The analysis of the Kingaroy data has been carried out in the same manner as the Nambour data.

It has been established that where there are breeding results for 2 years they do not differ significantly within mating groups. The breeding results have been pooled, therefore, within mating groups in further analysis of the data.

TABLE 4

SHOWING THE RESULTS OF 1166 INSEMINATIONS FROM EIGHT AIS BULLS DURING TWO BREEDING SEASONS IN THE KINGAROY AREA OF QUEENSLAND WITH RESPECT TO β-GLOBULIN TYPE OF COWS AND BULLS

Female phenotype	*Year*	*Male phenotype*															
		βAA				*βAD*				*βDD*				*βDE*			
		*Preg.**	*Ret'd†*	*Total*	%	*Preg.*	*Ret'd*	*Total*	%	*Preg.*	*Ret'd*	*Total*	%	*Preg.*	*Ret'd*	*Total*	%
βAA	1958	7	4	11		–	–	–		8	7	15		7	5	12	
	1959	–	–	–		17	17	34		6	4	10		–	–	–	
	Total	7	4	11	63·6	17	17	34	50·0	14	21	25	56·0	7	5	12	58·3
βAD	1958	33	40	73		–	–	–		46	40	86		57	67	124	
	1959	–	–	–		82	121	203		26	38	64		–	–	–	
	Total	33	40	73	45·2	82	121	203	40·4	72	78	150	46·7	57	67	124	45·9
βDD	1958	33	17	50		–	–	–		50	27	77		38	41	79	
	1959	–	–	–		71	64	135		21	15	36		–	–	–	
	Total	33	17	50	66·0	71	64	135	52·6	71	42	113	62·8	38	41	79	48·2
βAE	1958	3	4	7		–	–	–		6	4	10		1	2	3	
	1959	–	–	–		8	7	15		1	5	6		–	–	–	
	Total	3	4	7	42·8	8	7	15	53·3	7	9	16	43·7	1	2	3	33·3
βDE	1958	6	6	12		–	–	–		12	9	21		10	4	14	
	1959	–	–	–		21	24	45		3	4	7		–	–	–	
	Total	6	6	12	50·0	21	24	45	46·7	15	13	28	53·6	10	4	14	71·5
βEE	1958	2	0	2		–	–	–		1	1	2		1	1	2	
	1959	–	–	–		5	4	9		1	1	2		–	–	–	
	Total	2	0	2	100·0	5	4	9	55·6	2	2	4	50·0	1	1	2	50·0

* Pregnant. † Returned to service.

As with the Nambour data, some of the Kingaroy pooled breeding results differ significantly from the mean. However, the pooled data as a whole are not significantly heterogeneous ($\chi^2_{23} = 33{\cdot}89$, $0{\cdot}05 < P < 0{\cdot}1$). Leaving out the seventeen breeding results with β^{EE} cows, the breeding efficiencies in the residual twenty mating groups then show significant heterogeneity, due largely to the loss of four degrees of freedom in the χ^2 comparison ($\chi^2_{19} = 33{\cdot}39$, $P < 0{\cdot}02$). Because the heterogeneity is not due to variation in breeding results between years, it can be concluded that the Kingaroy data also reveal real differences in breeding efficiency between the β-globulin mating groups.

Examination of the pooled results suggested that the twenty-four mating groups could be rearranged on the same basis as the Nambour data. There is no evidence of a significant difference in breeding efficiency between the mating groups that have been combined. The mating groups were recombined as follows (bulls' phenotype first):

1. Unlike homozygotes, $\beta^{AA} \times \beta^{DD}$, $\beta^{DD} \times \beta^{AA}$, $\beta^{AA} \times \beta^{EE}$, $\beta^{DD} \times \beta^{EE}$.
   Mean breeding efficiency = 63·0%.
2. Like homozygotes, $\beta^{AA} \times \beta^{AA}$, $\beta^{DD} \times \beta^{DD}$.
   Mean breeding efficiency = 62·9%.
3. Homozygote × heterozygotes, $\beta^{AA} \times \beta^{AD}$, $\beta^{AA} \times \beta^{AE}$, $\beta^{AA} \times \beta^{DE}$, $\beta^{DD} \times \beta^{AD}$, $\beta^{DD} \times \beta^{AE}$, $\beta^{DD} \times \beta^{DE}$, $\beta^{AD} \times \beta^{AA}$, $\beta^{AD} \times \beta^{DD}$, $\beta^{AD} \times \beta^{EE}$, $\beta^{DE} \times \beta^{AA}$, $\beta^{DE} \times \beta^{DD}$, $\beta^{DE} \times \beta^{EE}$.
   Mean breeding efficiency = 49·5%.
4. Heterozygote × heterozygotes, $\beta^{AD} \times \beta^{AD}$, $\beta^{AD} \times \beta^{DE}$, $\beta^{AD} \times \beta^{AE}$, $\beta^{DE} \times \beta^{AD}$, $\beta^{DE} \times \beta^{AE}$, $\beta^{DE} \times \beta^{DE}$.
   Mean breeding efficiency = 44·3%.

TABLE 5

SIGNIFICANCE OF THE COMPARISON BETWEEN THE KINGAROY COMBINED-MATING-GROUPS MEAN BREEDING EFFICIENCIES BY WOOLF'S (1955) METHOD

Comparison	*Like homozygotes*	*Homozygote × heterozygotes*	*Heterozygote × heterozygotes*
Unlike homozygotes	(0·000) 0·017	(0·054) 4·544*	(0·284) 5·904*
Like homozygotes	–	(0·172) 6·598*	(0·004) 9·170**
Homozygote × heterozygotes	–		(0·321) 1·775

The χ^2 values shown are corrected for heterogeneity due to years. The heterogeneity χ^2 values are shown in parenthesis, none of the values being significant (three degrees of freedom).

* Significant at 5% level.
** Significant at 1% level.

Table 5 shows the corrected and heterogeneity χ^2 values for various comparisons between the combined mating groups. As with the analogous combined Nambour mating groups (Table 3), there is no significant difference in breeding efficiency between like-homozygote and unlike-homozygote matings, nor between homozygotes × heterozygote and heterozygote × heterozygote matings. But, there is a highly significant difference (corrected $\chi^2 = 12{\cdot}83$, $P < 0{\cdot}01$)

between matings involving only homozygotes (mean breeding efficiency = 62·9%) and matings in which heterozygotes are involved (mean breeding efficiency = 47·2%).

COMPARISON OF NAMBOUR AND KINGAROY BREEDING RESULTS

Apart from direct comparison between mating groups, either singly or in combination, the data presented may also be analysed according to the effect of bull or cow genotype on breeding efficiency. Table 6 shows corrected χ^2 values (by Woolf's procedure) for comparisons of this sort. As already noted, matings in each region between homozygous bulls and cows are very significantly superior to matings in which one or both are heterozygous. Also, in the Nambour region, inseminations by homozygous bulls are very significantly superior to inseminations by heterozygous bulls. The same effect is apparent in the Kingaroy region but is not significant. In the Kingaroy data, inseminations into homozygous cows irrespective of bull type are very significantly superior to inseminations into heterozygous cows. The same effect is seen in the Nambour data, but is not statistically significant.

TABLE 6

BREEDING EFFICIENCIES AND CORRECTED χ^2 VALUES FOR FURTHER COMPARISONS BETWEEN BREEDING RESULTS IN THE NAMBOUR AND KINGAROY DATA

Region	*Comparison*	*Breeding efficiency*	*Corrected* χ^2¶	*Heterogeneity* χ^2†
Nambour	Matings between homozygotes Matings involving heterozygotes	57·98 47·68	11·28***	1·86
Kingaroy	Matings between homozygotes Matings involving heterozygotes	62·93 47·24	12·83***	0·04
Nambour	Homozygous bulls Heterozygous bulls	54·77 43·57	12·44***	3·62
Kingaroy	Homozygous bulls Heterozygous bulls	53·97 47·11	2·14	1·37
Nambour	Homozygous cows Heterozygous cows	52·55 49·55	1·69	0·85
Kingaroy	Homozygous cows Heterozygous cows	56·30 45·65	12·50***	0·24

*** Significant at 0·1% level.
¶ One degree of freedom.
† Heterogeneity due to years.
Three degrees of freedom for Nambour, one for Kingaroy.

RETURNS TO SERVICE

The alternative to pregnancy in the data presented here is listed as a 'return to service'. The return of heat following insemination (which is a sign that insemination has failed and further service is necessary) may be due to one of two causes. Either insemination did not result in conception and the oestrous

TABLE 7

DISTRIBUTION OF RETURNS TO SERVICE FOR THE COMBINED MATING GROUPS OF TABLES 3 AND 5

Region	*Mating group*	*Return period in days*						*Total returns**
		1 *to* 17		18 *to* 24		25 *onwards*		
		No.	%	*No.*	%	*No.*	%	
Nambour	Like homozygotes	17	15·9	62	57·9	28	26·2	107
	Unlike homozygotes	9	14·1	45	70·3	10	15·6	64
	Homozygotes × heterozygotes	64	18·5	201	57·6	83	23·9	348
	Heterozygotes × heterozygotes	18	11·6	105	67·7	32	20·7	155
Kingaroy	Like homozygotes	5	11·4	32	72·7	7	15·9	44
	Unlike homozygotes	4	13·8	21	72·4	4	13·8	29
	Homozygotes × heterozygotes	30	14·2	135	63·6	47	22·2	212
	Heterozygotes × heterozygotes	26	14·1	119	64·3	40	21·6	185

* This total does not include a number of returns for which the time of return was not recorded.

cycle was not disrupted, or fertilization took place but the products of conception did not remain viable. In the former case, the great majority of the returns will fall into the period of the normal oestrous cycle, taken as 18 to 24 days. Returns longer than 24 days after insemination provide a crude guide to embryonic mortality, although an unknown proportion of these late returns is due to long cycle lengths. Table 7 shows the distribution of returns to service for the combined mating groups shown in Tables 3 and 6. The ratios of short, normal and long returns for the combined mating groups in both regions do not differ significantly from the mean ratios for the region.

DISCUSSION

BREEDING EFFICIENCIES AND RECIPROCAL MATING RESULTS

The search for a β-globulin effect on fertility was initiated by the observation that reciprocal matings between homozygotes and heterozygotes resulted in asymmetrical segregation ratios as judged from a survey of female progeny. From such matings, there was found to be a consistent excess of offspring of the mother's β-globulin genotype (termed for convenience the 'like-mother effect'). This occurred with four reciprocal mating groups in Friesian cattle and three groups (no matings being recorded in the fourth group) in Ayrshire cattle. The probability of this effect being spurious is less than 1 in 100 ($\chi^2_1 = 10{\cdot}4$, $P<0{\cdot}01$). A full interpretation of the effects of the serum β-globulins on fertility must take these findings into account, therefore.

Before the breeding efficiencies for the various mating groups were known, it was postulated that the asymmetrical segregation ratios were caused by embryonic mortality (Ashton, 1959), and that this was due to incompatability between mother and foetus of different β-globulin genotype. The data presented here were collected specifically to test this hypothesis. The artificial-breeding data show that matings between unlike homozygotes are as fertile as matings between like homozygotes. This renders untenable the simple postulate that unlike-mother zygotes are less viable than those like mother, because all the embryos from like homozygotes will be like the mother and all those from unlike homozygotes unlike the mother. If one tries to rationalize the hypothesis, some compensating mechanism would have to be postulated to explain this. Because the like-homozygote and unlike-homozygote breeding percentages are not significantly different, the compensating effect would have to be of the same order of magnitude as the 'like-mother effect'. It would be necessary to postulate that heterozygous embryos *per se* are at an advantage *in utero* and that this would compensate for their being unlike-mother. If this were so, there would be no excess of like-mother offspring from the matings $\beta^{AD}\sigma \times \beta^{AA}\varphi$ or $\beta^{AD}\sigma \times \beta^{DD}\varphi$, for the number of heterozygous embryos lost through being unlike mother should then be compensated by the number of homozygous embryos lost through not being heterozygous.

Whatever the reason for the aberrant segregation ratios with female offspring, there is no evidence of differential embryonic mortality between β-globulin mating groups as postulated (Table 7). To pursue the subject further, it would be necessary to investigate herds in which artificial insemination is practised

and in which all offspring born, both male and female, are kept. Although such herds do not exist in sufficient numbers at the present time, projected experiments with artificial insemination of beef cattle in Queensland may provide the necessary data.

β-GLOBULIN MATING GROUP AND BREEDING EFFICIENCY

Leaving aside the confusing and possibly irrelevant asymmetrical segregation ratios, the results leave little doubt that the β-globulin types of the parents influence the breeding percentage. The concordance between the Nambour and Kingaroy data adds considerable weight to this conclusion. In addition, similar differences have been found by Ogden (1960) working with cattle typed during a survey of β-globulin gene frequencies in British dairy breeds (Ashton, 1958).

Matings between homozygotes are more fertile than matings in which one or two heterozygotes are involved. This result, by itself, could be readily explained by postulating, for example, that heterozygous parents produce fewer gametes. Alternatively, the chances of the gametes being able to fuse might be modified by the β-globulin genotype of the parent, irrespective of the β-globulin allele carried by the gamete. Postulates of this type should be amenable to experimental verification.

The effect of the serum β-globulin types of the parents on breeding efficiency is quite large. Reference to Table 6 shows that the possible increase in breeding percentage over the mean of about 50% by selective mating of homozygous bulls and cows is about 8% (Nambour) to 13% (Kingaroy). Taking the mean (10%), this implies that eighty-three inseminations only would be required to produce the same results as currently obtained with 100 inseminations in these regions. This would mean a worthwhile financial saving in the operation of an artificial-breeding service. It would also mean that the farmer would have fewer cows out of production in any given period. As it is unlikely that herds of homozygous cows could be readily established, it is suggested that artificial-breeding centres should type all bulls being considered for A.I. and select only homozygous bulls.

A complete change-over to homozygous bulls would increase the average breeding percentages about 4%, giving a corresponding reduction in inseminations of about 7 to 8%. It is worth noting that the extent of the gain in fertility will depend on the number of bulls in use at the centre. If only a few bulls are kept they may well be, by chance, all heterozygous.

Either β^{AA} or β^{DD} bulls could be chosen, β^{EE} bulls being very rare in the common British dairy breeds. It has already been shown (Ashton, 1960) that milk yield is non-randomly distributed with respect to β-globulin type. Thus, β^{DD} bulls at both Milk Marketing Board and privately-owned cattle-breeding centres in England and Wales had significantly superior mean contemporary comparison values to β^{AA} bulls, being about 25 gal. better. For increasing milk yield and fertility, the logical choice is for β^{DD} bulls. However, it should be remembered that in one survey β^{AE} and possibly β^{DE} Ayrshire cows gave superior butterfat percentages in their first lactation (Ashton, 1960), although

the effect was not statistically significant. When the selection programme is aimed at increasing the butterfat percentage of the milk, the gain in fertility resulting from the use of β^{DD} bulls might be offset by the lowered butterfat percentage of non-β^{E} cows. Curiously, the high-fat-producing breeds (Jersey, Guernsey and South Devon) do not have β^{E}, and the problem would not arise. Further data on the relationship between butterfat percentage and β-globulin type are being collected in herds of AIS cows.

STATUS OF β-GLOBULIN HETEROZYGOTES

The findings reported here represent a curious genetic situation, because heterozygotes would appear to be at a disadvantage with regard to fertility. However, it has been found in several instances that the number of heterozygotes in random samples from unrelated cow populations is greater than expected from the calculated gene frequencies. The same effect has also been observed by Gahne, Rendel & Venge (1960). It seems probable that heterozygotes are more viable, therefore, and that this heterozygote superiority would be sufficient to produce a stable polymorphism. This subject will be discussed in more detail in a future publication.

ACKNOWLEDGMENTS

I wish to thank Messrs G. R. Fallon and D. Sutherland of the Queensland Department of Agriculture and Stock for helpful cooperation in organizing the bleeding of the cows and supplying the breeding data. Also Messrs J. M. Crofts and J. T. Piggott at the Nambour and Kingaroy sub-centres, respectively, for providing blood samples from cows, and J. W. Ryley at the Animal Research Institute, Yeerongpilly, Brisbane, for providing blood samples from bulls. I am indebted to Mr H. G. Turner and Dr J. M. Rendel for constructive criticism of the manuscript, and to C. Bloomfield for technical assistance.

REFERENCES

ASHTON, G. C. (1957) Serum protein differences in cattle by starch gel electrophoresis. *Nature, Lond.* **180**, 917.

ASHTON, G. C. (1958) Genetics of β-globulin polymorphism in British cattle. *Nature, Lond.* **182**, 370.

ASHTON, G. C. (1959) β-globulin polymorphism and early foetal mortality in cattle. *Nature, Lond.* **183**, 404.

ASHTON, G. C. (1960) β-globulin polymorphism and economic factors in dairy cattle. *J. agric. Sci.* **54**, 321.

FALLON, G. R. (1958) Artificial insemination of dairy cattle in Queensland. *Proc. aust. Soc. Anim. Prod.* **2**, 90.

GAHNE, B., RENDEL, J. & VENGE, O. (1960) Inheritance of β-globulins in serum and milk from cattle. *Nature, Lond.* **186**, 907.

OGDEN, A. L. (1960) β-globulin type and conception rate in artificially bred dairy cattle. *Rep. VII^th Europ. Meet. on Blood Groups in Farm Anim., Edinburgh*, p.150

SMITHIES, O. (1955) Zone electrophoresis in starch gels. *Biochem. J.* **61**, 629.

WOOLF, B. (1955) On estimating the relation between blood group and disease. *Ann. hum. Genet.* **19**, 251.

Commonwealth of Australia,
Commonwealth Scientific and Industrial Research Organization.

Reprinted from *J. Reprod. Fertil.* (1962) **3**, 93-104

β-GLOBULIN TYPE, FERTILITY AND EMBRYONIC MORTALITY IN CATTLE

G. C. ASHTON AND G. R. FALLON

Cattle Research Laboratory, Commonwealth Scientific and Industrial Research Organization, Rockhampton, Queensland, and Veterinary School, University of Queensland, Brisbane, Queensland, Australia,

(*Received 6th May* 1961)

Summary. It has been reported previously that serum β-globulin polymorphism affects fertility in dairy cattle, matings between homozygotes being significantly more fertile than matings involving heterozygotes. Indirect methods have been used to determine whether this is due to differential mortality of the embryonic genotypes, or to differences in fertilization efficiency.

From a study of six unrelated dairy-cattle populations and a closed beef-cattle population, it was shown that an excess of heterozygotes is born. The distribution of returns 25 days or longer after artificial insemination, used as an index of embryonic death, as well as the distribution of genotypes from known matings, support the conclusion that homozygotes are less viable than heterozygotes *in utero*. The distribution of returns 0 to 24 days after insemination, however, showed that matings between homozygous parents of like genotype have a greater chance of achieving fertilization than matings between homozygous parents of unlike genotype.

It is concluded that the β-globulin locus in cattle affects fertility in two ways, at fertilization, and *in utero*.

INTRODUCTION

Data relating to bovine fertility have been accumulated by artificial insemination centres in the major dairying countries. In all countries, it has been observed that the overall breeding efficiency obtained increases as experience with the technique is gained, but that eventually little further improvement occurs. The point at which this happens varies between countries but a breeding efficiency between 65 and 70% has been commonly reported.

The reasons for this are appreciated but not understood. It is known for example that some inseminations are ineffective because they are performed when the cow is not in oestrus or because the semen is too old. However, even with cows known to have been inseminated at oestrus with fertile semen, some 10% of inseminations (Robinson, 1957) do not result in conception. An even

greater loss is due to embryonic and foetal mortality, 25 to 30% of successful conceptions failing within 3 months.

The 'long returns' index, that is the percentage of animals inseminated during oestrus which show their next heat 25 days or more after the insemination, is sometimes taken as a measure of embryonic death. Various authors (Ogden, 1959; Boyd & Reed, 1961) have shown that this index is relatively constant irrespective of whether it is calculated from first or repeat inseminations. Ogden suggested that embryonic mortality may be largely genetic in character, and its rate fixed by the random character of mating between genotypes usual in artificial insemination practice.

Recently, a simply inherited genetic factor has been shown to influence fertility in cattle (Ashton, 1961). Matings between cows and bulls homozygous at the serum β-globulin locus have proved to be on average significantly more fertile than matings between individuals one or both of which is heterozygous at this locus. This has been demonstrated in both Jersey and Australian Illawarra Shorthorn cattle in Queensland, and (A. L. Ogden, personal communication) in some dairy breeds in Great Britain.

Indirect methods must be used in cattle to determine whether parental β-globulin type affects embryonic and foetal loss or the chance of fertilization. Two independent methods have been employed. Firstly, the proportion of homozygotes and heterozygotes conceived from different matings has been compared with the proportion born. Secondly, the percentage of returns 25 days or longer after insemination has been used as a crude index of late embryonic and early foetal mortality and the percentage of returns 0 to 24 days after insemination as an estimate of fertilization efficiency plus early embryonic loss.

MATERIAL AND METHODS

MATERIALS

Serum samples have been obtained from dairy cattle in several surveys, and from beef cattle at the National Cattle Breeding Station, as follows:

(1) From 179 Friesian and 139 Ayrshire cows selected at random from herds using artificial insemination in England and Wales.

(2) From 90 Friesian cows and 113 of their female progeny from a large herd not employing artificial insemination in East Anglia.

(3) From 675 Jersey cows in the bull-progeny-testing scheme in the Nambour region of Queensland. These were the dams providing the breeding results reported previously (Ashton, 1961).

(4) From 622 Australian Illawarra Shorthorn cows in the bull-progeny-testing scheme in the Kingaroy region of Queensland, reported in part previously (Ashton, 1961).

(5) From beef Shorthorn and Hereford cows and bulls and their progeny, and from beef Shorthorn and Hereford cows and Africander bulls and their progeny at the National Cattle Breeding Station, 'Belmont', Rockhampton, Queensland. These animals form part of a project on genetics of adaptation in cattle (Kennedy & Turner, 1959).

DETERMINATION OF SERUM β-GLOBULIN TYPE

The procedure used has been described previously (Ashton, 1958a, 1960, 1961).

ARTIFICIAL INSEMINATION DATA

These were supplied by the Queensland Department of Agriculture and Stock, and were obtained during the course of the Nambour and Kingaroy bull-proving projects (Fallon, 1958a).

RESULTS

DISTRIBUTION OF β-GLOBULIN TYPES IN RANDOM SAMPLES OF DAIRY COWS

Table 1 shows the distribution of the β-globulin types of dairy cows in several unrelated populations. In the case of the East Anglian Friesian herd not using artificial insemination, the data relate to cows and their female progeny, the majority of which were milking at the time of sampling. From the genotypes of the individuals in each population, the gene frequencies were calculated by a maximum-likelihood method (Ashton, 1958 a, b). From these derived gene frequencies, the expected number of each β-globulin genotype in each population was calculated assuming the population represented a Hardy-Weinberg distribution. The expected number was compared with the number of that type observed in the sample with the χ^2 test.

In each sample, with the exception of the AI Friesian cows there is an excess of observed compared with expected heterozygotes and a concomitant lack of observed homozygotes. The excess is only significant for the Australian Illawarra Shorthorn cows, which was the largest population studied. There is an excess of observed homozygotes in the AI Friesian cows but it is not significant. Overall, there is a significant excess of observed heterozygotes. Gahne, Rendel & Venge (1960) have also reported an excess of heterozygotes in a sample of 707 Swedish Red and White (S.R.B.) cattle but this was not significant ($\chi^2 = 2{\cdot}75$, $0{\cdot}05 < P < 0{\cdot}1$).

The data suggest that a typical 'random' population of dairy cows might have a preponderance of heterozygous β-globulin types compared with the distribution expected from the derived gene frequencies.

DISTRIBUTION OF β-GLOBULIN TYPES OF OFFSPRING FROM BEEF-CATTLE MATINGS

An excess of heterozygotes in a cow population could originate in more than one way. It is difficult to distinguish between these possibilities with dairy cattle because most male calves are disposed of soon after birth, nor is it usually possible to sample the culled female progeny. The position is different with beef cattle, where male and female progeny are both commercially valuable. Unfortunately, very few beef herds keep accurate pedigree records. An exception is the National Cattle Breeding Station, 'Belmont', Rockhampton, Queensland. This herd is closed, and the pedigree of all progeny is available. Post-natal losses are low and accordingly progeny sampled at any time up to disposal reflect the distribution obtained from parents of known β-globulin

Table 1

OBSERVED AND EXPECTED DISTRIBUTION OF β-GLOBULIN GENOTYPES FROM 1518 COWS OF DIFFERING ORIGIN AND BREED, AND COMPARISON OF THE OBSERVED AND EXPECTED NUMBERS OF HOMOZYGOTES AND HETEROZYGOTES

Sample	*Observed or expected*	*Distribution of genotypes*							*Comparison*			
		Total	*AA*	*AD*	*DD*	*AE*	*DE*	*EE*	*Total homozygotes*	*Total heterozygotes*	*Significance*	
											χ^2	*P*
AI Cows, England and Wales*												
Friesian	Obs.	179	54	72	42	7	4	0	96	83	2·81	n.s.
	Exp.	179	48·8	83·5	35·8	5·8	4·9	0·2	84·8	94·2		
Ayrshire	Obs.	139	6	45	43	10	31	4	53	86	1·28	n.s.
	Exp.	139	8·1	39·1	47·2	11·8	28·5	4·3	59·6	79·4		
Non-AI Cows, East Anglia												
Friesian	Obs.	203	21	102	76	3	1	0	97	106	1·87	n.s.
	Exp.	203	26·6	92·3	80·1	1·5	2·5	0	106·7	96·3		
AI Cows, Queensland												
Jersey	Obs.	375	104	199	72	0	0	0	176	199	1·78	n.s.
	Exp.	375	110·6	186·1	78·3	—	—	—	188·9	186·1		
Illawarra Shorthorn	Obs.	622	35	262	221	24	70	10	266	356	6·11	< 0·01
	Exp.	622	50·9	221·3	240·6	32·7	71·2	5·3	296·8	323·2		
Totals	Obs.								688	830	6·28	< 0·02
	Exp.								736·8	781·2		

* Artificially inseminated.

type. The distribution of progeny for the British breeds on 'Belmont' (Hereford × Hereford, Shorthorn × Shorthorn, and Hereford × Shorthorn) for the years 1955-58 is shown in Table 2, together with the distribution from the progeny of Africander bulls and Hereford and Shorthorn cows during the same period. The progeny for a few F2 matings (Africander Hereford × Africander Hereford and Africander Shorthorn × Africander Shorthorn) are included.

TABLE 2

DISTRIBUTION OF HOMOZYGOUS AND HETEROZYGOUS OFFSPRING FROM SOME MATINGS BETWEEN (*a*) BRITISH BREED COWS AND BULLS, AND (*b*) BRITISH BREED COWS AND AFRICANDER BULLS, DURING THE PERIOD 1955-1958 AT THE NATIONAL CATTLE BREEDING STATION, ROCKHAMPTON, QUEENSLAND

Parents' genotype	*Genotype of offspring*							
	(*a*) *British breeds*				(*b*) *Africander × British*			
	Homozygotes		*Heterozygotes*		*Homozygotes*		*Heterozygotes*	
Bull Cow	*Obs.*	*Exp'd*	*Obs.*	*Exp'd*	*Obs.*	*Exp'd*	*Obs.*	*Exp'd*
AA × AD	0	0	0	0	0	3·0	6	3·0
AD × AA	13	15·5	18	15·5	2	6·0	10	6·0
DD × AD	10	12·5	15	12·5	0	0	0	0
AD × DD	22	24·5	27	24·5	0	0	0	0
AA × AE	0	0	0	0	1	1·0	1	1·0
AE × AA	0	0	0	0	7	8·0	9	8·0
AD × AE	2	2·75	9	8·25	2	2·5	8	7·5
AE × AD	0	0	0	0	7	12·5	43	37·5
AD × DE	1	2·0	7	6·0	0	2·5	10	7·5
DE × AD	0	1·0	4	3·0	5	2·75	6	8·25
DE × AE	0	0·5	2	1·5	1	1·0	3	3·0
AE × DE	0	0	0	0	0	0·5	2	1·5
DD × DE	1	1·0	1	1·0	0	0	0	0
DE × DD	1	1·0	1	1·0	2	1·0	0	1·0
AD × AD	29	30·5	32	30·5	7	5·0	3	5·0
AE × AE	0	0	0	0	0	2·0	4	2·0
DE × DE	0	0	0	0	0	0·25	1	0·75
Totals	79	91·25	116	103·75	34	48·0	106	92·0
Significance	$\chi^2 = 3{\cdot}09$; $0{\cdot}05 < P < 0{\cdot}1$				$\chi^2 = 6{\cdot}21$; $P < 0{\cdot}02$			

Table 2 shows an excess of observed compared with expected heterozygotes and a corresponding lack of homozygotes for both breed groups. In the case of the Africander × British progeny, the excess is significant. The excess of heterozygotes in the progeny from these matings supports the excess of heterozygotes found in random samples of cows. It also shows that the excess is probably due to an excess born, and not to differential viability or heterozygote selection after birth.

PATTERN OF RETURNS-TO-SERVICE

The dioestrous interval in non-inseminated cows averages 21 days. In Jersey cows in Queensland, about 70% of such intervals are between 18 and 25 days duration, about 18% are 'short' cycles, 0 to 17 days duration, and about 12% are 'long' cycles, 26 days or more in duration (Fallon, 1958b). By contrast, the distribution of the comparable interval, called the return-to-service interval, in cows inseminated at oestrus is quite different. In the data reported here, 14% of these return-to-service intervals are in the range 0 to 17 days, and may be termed conveniently 'short' returns; 55% of the returns are in the range 18 to 24 days, and may be termed 'normal' returns; and 31% are 'long' returns occurring 25 days or more after insemination. The relative increase in long returns in inseminated cows compared with the percentage of long cycles in non-inseminated cows is due either to death of embryos before or about the

TABLE 3

DISTRIBUTION OF RETURNS-TO-SERVICE IN THE COMBINED JERSEY AND A.I.S. INSEMINATION DATA FOR THE NINE POSSIBLE MATINGS INVOLVING THE ALLELES β^A AND β^D

Mating group		*Total No. insemina-tions*	*No. pregnant*	*Breeding efficiency* (%)	*Returns*		*Returns 0 to 24 days* (%)	*Returns 25 days or more* (%)
Bull	*Cow*				*0 to 24 days*	*25 days and over*		
AA	AA	256	143	55·9	74	39	28·9	15·2
AA	AD	520	265	51·0	174	81	33·5	15·6
AA	DD	231	143	61·9	69	19	29·9	8·2
AD	AA	214	97	45·3	77	40	36·0	18·7
AD	AD	570	260	45·6	217	93	38·1	16·3
AD	DD	342	172	50·3	114	56	33·3	16·4
DD	AA	42	25	59·5	13	4	31·0	9·5
DD	AD	209	98	46·9	75	36	35·9	17·2
DD	DD	170	94	55·3	57	19	33·5	11·2
Totals and averages		2544	1287	50·6				

time of implantation at approximately 35 days or to death of foetuses. The percentage of inseminations causing long returns consequently is sometimes used as a crude index of late embryonic and early foetal death (Robinson, 1957; Ogden, 1959; Ashton, 1961).

The interpretation of such an index, however, is not easy. The developing young may die any time after insemination. If the zygote ceases development before the corpus luteum is affected, which is thought to be about 16 days after fertilization, oestrus will return at the end of the normal period as if fertilization had not occurred. Robinson (1957) terms this early embryonic death. The developing embryo subsequent to this stage may die before implantation (late embryonic death). Because the corpus luteum will have become a corpus luteum of pregnancy, the onset of the next oestrous cycle will be delayed accordingly. Similarly, death after implantation (foetal death) will also delay the return-to-service.

Any factor causing increased late embryonic or foetal death will increase the percentage of long returns. However, because the time distribution of late embryonic death overlaps the normal dioestrous interval, there will also be an increase in the normal returns. Conversely an increase in normal returns could result from a decrease in fertilization efficiency or an increase in early embryonic death. Either event would also tend to increase long returns because of the previously described distribution of dioestrous intervals. Clearly, returns-to-service data must be interpreted with caution.

Table 3 shows the distribution of returns-to-service following 1527 inseminations from Jersey bulls into nominally Jersey cows, and 1017 inseminations from Australian Illawarra Shorthorn (A.I.S.) bulls into nominally A.I.S. cows. The results for each mating for each breed have been combined, the results for comparable matings in the two breeds being very similar. Because g^E is not represented in Jersey cattle, matings involving β^E in the A.I.S. data

TABLE 4

VALUES OF χ^2 OBTAINED BY COMPARING DIFFERENT MATING GROUPS WITH REGARD TO RETURNS IN THE TWO PERIODS 0 TO 24 DAYS, AND 25 DAYS OR MORE, AFTER INSEMINATION

Mating groups compared				*Returns, days after insemination*	
I		II		0 *to* 24	25 *or more*
Bull	*Cow*	*Bull*	*Cow*		
AA ×	DD	DD ×	AA	0·20	0·08
AA ×	AA	DD ×	DD	1·03	1·43
AA ×	AD	AD ×	AA	0·43	0·06
AD ×	DD	DD ×	AD	0·37	0·07
AD ×	DD	AA ×	AD		
plus		plus		0·00	0·00
DD ×	AD	AD ×	AA		

None of the χ^2 values are significant.

are not included in Table 3. Further, only two time distributions for returns-tot service are shown, the significance of short returns as a separate entity no-being very clear. Short and normal returns are treated as one distribution, i.e. returns in the period 0 to 24 days, while long returns and returns to an anoestrous state are grouped as returns in the period 25 days or longer.

A number of derivations are possible: for example, the percentage of total inseminations giving long returns, the percentage giving normal returns, the percentage causing 'conception' as judged by the sum of long returns and pregnancies, the percentage of such 'conceptions' giving long returns, and so on. For the reasons discussed above, none of these derivations gives an unequivocal measure of fertilization efficiency or embryonic loss. For this reason, only two are considered further, the percentage of inseminations giving normal returns as a guide to fertilization efficiency and early embryonic death, and the percentage causing long returns as a guide to late embryonic and foetal death.

Table 3 shows that the two possible matings between unlike homozygotes, that is AA ♂ × DD ♀ and DD ♂ × AA ♀, give very similar results for breeding efficiency, returns in the 0- to 24-day period and long returns. The homogeneity of the breeding efficiency results for these and other combined mating groups has been examined more exhaustively in a previous publication (Ashton, 1961). The homogeneity of the returns in both the 0- to 24-day period and the long returns, for the two reciprocal matings between unlike homozygotes was tested by calculating χ^2 (Table 4). No significant difference between the AA ♂ × DD ♀ and DD ♂ × AA ♀ mating groups was found. The data for the two mating groups were combined therefore to give an 'unlike-homozygotes' mating group (Table 5).

In the same way, no significant differences in breeding efficiency, 0- to 24-day returns, or long returns were found in comparing the AA ♂ × AA ♀ and DD ♂ × DD ♀ mating groups (Table 4). The data for these mating groups were combined to give a 'like-homozygotes' mating group (Table 5).

The reciprocal mating groups AA ♂ × AD ♀ and AD ♂ × AA ♀ were

TABLE 5

BREEDING EFFICIENCY AND PERCENTAGE OF RETURNS IN THE TWO PERIODS FOR THE FOUR COMBINED MATING GROUPS

Combined mating groups	*Breeding efficiency* (%)	*Returns*	
		0 *to* 24 *days* (%)	25 *days or more* (%)
Unlike homozygotes	61·6	30·0	8·4
Like homozygotes	55·6	30·8	13·6
Homozygotes × heterozygotes	49·2	34·2	16·6
Heterozygotes × heterozygotes	45·6	38·1	16·3

found to give comparable breeding efficiencies and returns distributions, as were the reciprocal mating groups AA ♂ × AD ♀ and AD ♂ × DD ♀. Comparison of the combined AA × AD matings with the combined DD × AD matings did not reveal any significant differences. Although these groups can be considered in several different combinations, they have been treated as one combined mating group, homozygotes × heterozygotes.

The fourth combined mating group, heterozygotes × heterozygotes (Table 5), consists only of AD ♂ × AD ♀ matings.

Comparison of the four combined mating groups, one with the other with regard to both return periods by the χ^2 test, is shown in Table 6. From this Table, it is apparent that matings between unlike homozygotes differ significantly from matings between like homozygotes, between homozygotes and heterozygotes, and between heterozygotes in the percentage of returns occurring on or after the 25th day. On the other hand, matings between heterozygotes differ significantly from matings between unlike homozygotes or between like homozygotes in the percentage of returns occurring in the period 0 to 24 days. Matings between like homozygotes and between homozygotes and heterozygotes do not

differ significantly in either return period, nor do matings between heterozygotes and matings between homozygotes and heterozygotes.

DISCUSSION

The most likely explanation of the excess of heterozygotes from those matings where both homozygous and heterozygous offspring are expected in definable

TABLE 6

VALUES OF χ^2 OBTAINED BY COMPARING THE COMBINED MATING GROUPS SHOWN IN TABLE 5 WITH REGARD TO RETURNS IN THE TWO PERIODS, 0 TO 24 DAYS, AND 25 DAYS OR MORE, AFTER INSEMINATION

Combined mating groups compared		*Returns, days after insemination*	
I	II	0 *to* 24	25 *or more*
Unlike homozygotes	Like homozygotes	0·04	4·37*
Unlike homozygotes	Homozygotes × heterozygotes	2·01	11·93***
Unlike homozygotes	Heterozygotes × heterozygotes	5·21*	9·69**
Like homozygotes	Homozygotes × heterozygotes	2·02	2·28
Like homozygotes	Heterozygotes × heterozygotes	5·75*	1·38
Homozygotes × heterozygotes	Heterozygotes × heterozygotes	2·18	0·04

* Significant at 5% level
** Significant at 1% level
*** Significant at 0·1% level

proportion (Table 2), is that homozygous embryos are less likely to survive *in utero*. The extent of this loss may be judged from Table 2. The total number of homozygotes from matings where equal numbers of homozygotes and

TABLE 7

SHOWING THE ACTUAL NUMBERS OF HOMOZYGOTES AND HETEROZYGOTES OBSERVED, AS A PERCENTAGE OF EXPECTED, FOR THE DATA IN TABLE 1 AND FOR THE DATA OF GAHNE, RENDEL & VENGE (1960)

Sample	$\frac{Observed}{Expected} \times 100\%$		*Relative difference* (%)
	Homozygotes	*Heterozygotes*	
AI Ayrshire	88·9	108·3	19·4
Non-AI Friesian	90·9	110·1	19·2
Jersey	93·2	106·9	13·7
Illawarra Shorthorn	89·6	110·1	20·5
Gahne *et al.*	91·8	104·7	12·9
Mean	91·1	107·1	16·0

heterozygotes would be expected is ninety-nine whereas the corresponding number of heterozygotes is 119. This represents a loss of 16·8% of homozygotes. An independent, but less direct, estimate may be obtained by comparison of the expected and observed numbers of homozygotes and heterozygotes in dairy herds (Table 1). Table 7 shows the percentage of observed homozygotes

and heterozygotes in terms of the expected values. The A1 Friesians in Table 1 have been excluded because the results are anomalous, but the results of Gahne, *et al.* (1960) are included. There are on average 7·1% more heterozygotes than expected and 8·9% fewer homozygotes, giving a relative deficiency of homozygotes of 16·0%.

The agreement between the two estimates suggests that a reasonable value for homozygote loss has been obtained, approximately one in every six conceived β-globulin homozygotes being lost through embryonic or foetal mortality. A loss of this magnitude should be reflected in a comparison of the percentage of long returns for matings between unlike homozygotes from which all the zygotes will be heterozygotes and matings between like homozygotes from which all the zygotes will be homozygotes. Table 5 shows that matings between unlike homozygotes give 8·4% long returns, and matings between like homozygotes 13·6% long returns, a significant difference (Table 6). This is a difference in long returns of only 5·2%, about one-third of the expected difference of about 16%. The remaining 11% loss of homozygotes must occur, then, in the early embryonic death stage, up to about 16 days after conception, at a time when the normal cycle length is not interrupted. The percentage of normal returns is the same for the two homozygous mating groups. The postulated increase of 11% in normal returns in matings between like homozygotes must therefore be balanced by a loss of about 11% in normal returns in matings between unlike homozygotes. This could be a fertilization loss or an early embryonic mortality loss. To account for the differential between number of homozygotes and heterozygotes born, however, it must be a fertilization loss. An early embryonic mortality loss would lead to fewer heterozygotes being born, and thus lessen the differential between the relative number of homozygotes and heterozygotes in the adult population.

It can be postulated, in matings between homozygotes at least, that homozygous embryos are less likely to survive than heterozygous embryos, but matings between homozygous partners of the same genotype are more likely to result in fertilization than matings between homozygotes of differing genotype. However, a satisfactory hypothesis to explain the effects of β-globulin type on fertility must embrace the results of matings between homozygotes and heterozygotes, and between heterozygotes. From these matings an equal number of homozygous and heterozygous embryos, equally distributed between homozygous and heterozygous mothers, would be expected. A hypothesis that presupposes that homozygous embryos are at a disadvantage to heterozygous embryos with regard to viability *in utero*, irrespective of maternal genotype, requires a level of long returns intermediate between that for matings between like homozygotes and that for matings between unlike. Table 5 shows that this is not the case. The percentage of long returns for both mating groups involving heterozygotes is greater than that for matings between like homozygotes. There is also an increase in the percentage of normal returns for mating involving heterozygotes, normal returns for matings between heterozygotes being significantly greater than those for matings between homozygotes, either like or unlike (Table 6).

It is not possible to determine solely from the returns data whether the

increased normal returns in matings between homozygotes and heterozygotes compared with matings between like homozygotes are due to early embryonic mortality or to decreased fertilization. It is likely that an increase in early embryonic mortality would be accompanied by an increase in late embryonic mortality, thus accounting for the relative increase in late returns above the intermediate value expected. The nature of a factor that could cause increased embryonic loss from matings between homozygotes and heterozygotes is not known.

Direct proof of the hypothesis that matings between like homozygotes are more likely to achieve fertilization than matings between unlike homozygotes, and that heterozygous embryos are more likely to succeed *in utero* than homozygotes, will be difficult with cattle for practical reasons. A more amenable animal will have to be found in which β-globulin polymorphism exists, and in which the β-globulin effect on fertility is operating. All mammals so far examined have shown this polymorphism and it is unlikely that the effect on fertility found in dairy cattle is unique. Mice show the polymorphism (Ashton & Braden, 1961; Cohen, 1960; Shreffler, 1960), but it is a rather specialized kind in which all but one strain so far examined are homozygous for one allele and the remaining strain is homozygous for another allele. No naturally occurring heterozygotes have been found. β-globulin polymorphism has also been observed in other laboratory animals (Ashton, 1958b) and one of these species may prove suitable.

ACKNOWLEDGMENTS

None of these data could have been collected without the active and generous co-operation of individual members of the Milk Marketing Board of England and Wales, the Queensland Department of Agriculture and Stock, and the National Cattle Breeding Station, 'Belmont', Rockhampton, Queensland. Too numerous to mention individually we are glad to acknowledge their valuable help. We are indebted to Dr J. M. Rendel and Mr H. G. Turner for constructive criticism of the manuscript, and to C. Bloomfield for technical assistance.

REFERENCES

Ashton, G. C. (1958a) Genetics of β-globulin polymorphism in British cattle. *Nature, Lond.* **182**, 370.

Ashton, G. C. (1958b) *Polymorphism in the serum proteins of mammals.* Ph.D. Thesis, University of Liverpool.

Ashton, G. C. (1960) β-globulin polymorphism and economic factors in dairy cattle. *J. agric. Sci.* **54**, 321.

Ashton, G. C. (1961) β-globulin type and fertility in artificially bred dairy cattle. *J. Reprod. Fertil.* **2**, 117.

Ashton, G. C. & Braden, A. W. H. (1961) β-globulin polymorphism in laboratory mice. *Aust. J. biol. Sci.* **14**, 248.

Boyd, H. & Reed, H. C. B. (1961) Investigations into the incidence and causes of infertility in dairy cattle—Fertility variations. *Brit. vet. J.* **117**, 18.

Cohen, B. L. (1960) Genetics of plasma transferrins in the mouse. *Genet. Res.* **1**, 431.

Fallon, G. R. (1958a) Artificial insemination of dairy cattle in Queensland. *Proc. Aust. Soc. Anim. Prod.* **2**, 90.

Fallon, G. R. (1958b) Some aspects of oestrum in cattle, with reference to fertility in artificial insemination. *Qld. J. agric. Sci.* **15**, 25.

and heterozygotes in terms of the expected values. The AI Friesians in Table 1 have been excluded because the results are anomalous, but the results of Gahne, *et al.* (1960) are included. There are on average 7·1% more heterozygotes than expected and 8·9% fewer homozygotes, giving a relative deficiency of homozygotes of 16·0%.

The agreement between the two estimates suggests that a reasonable value for homozygote loss has been obtained, approximately one in every six conceived β-globulin homozygotes being lost through embryonic or foetal mortality. A loss of this magnitude should be reflected in a comparison of the percentage of long returns for matings between unlike homozygotes from which all the zygotes will be heterozygotes and matings between like homozygotes from which all the zygotes will be homozygotes. Table 5 shows that matings between unlike homozygotes give 8·4% long returns, and matings between like homozygotes 13·6% long returns, a significant difference (Table 6). This is a difference in long returns of only 5·2%, about one-third of the expected difference of about 16%. The remaining 11% loss of homozygotes must occur, then, in the early embryonic death stage, up to about 16 days after conception, at a time when the normal cycle length is not interrupted. The percentage of normal returns is the same for the two homozygous mating groups. The postulated increase of 11% in normal returns in matings between like homozygotes must therefore be balanced by a loss of about 11% in normal returns in matings between unlike homozygotes. This could be a fertilization loss or an early embryonic mortality loss. To account for the differential between number of homozygotes and heterozygotes born, however, it must be a fertilization loss. An early embryonic mortality loss would lead to fewer heterozygotes being born, and thus lessen the differential between the relative number of homozygotes and heterozygotes in the adult population.

It can be postulated, in matings between homozygotes at least, that homozygous embryos are less likely to survive than heterozygous embryos, but matings between homozygous partners of the same genotype are more likely to result in fertilization than matings between homozygotes of differing genotype. However, a satisfactory hypothesis to explain the effects of β-globulin type on fertility must embrace the results of matings between homozygotes and heterozygotes, and between heterozygotes. From these matings an equal number of homozygous and heterozygous embryos, equally distributed between homozygous and heterozygous mothers, would be expected. A hypothesis that presupposes that homozygous embryos are at a disadvantage to heterozygous embryos with regard to viability *in utero*, irrespective of maternal genotype, requires a level of long returns intermediate between that for matings between like homozygotes and that for matings between unlike. Table 5 shows that this is not the case. The percentage of long returns for both mating groups involving heterozygotes is greater than that for matings between like homozygotes. There is also an increase in the percentage of normal returns for mating involving heterozygotes, normal returns for matings between heterozygotes being significantly greater than those for matings between homozygotes, either like or unlike (Table 6).

It is not possible to determine solely from the returns data whether the

increased normal returns in matings between homozygotes and heterozygotes compared with matings between like homozygotes are due to early embryonic mortality or to decreased fertilization. It is likely that an increase in early embryonic mortality would be accompanied by an increase in late embryonic mortality, thus accounting for the relative increase in late returns above the intermediate value expected. The nature of a factor that could cause increased embryonic loss from matings between homozygotes and heterozygotes is not known.

Direct proof of the hypothesis that matings between like homozygotes are more likely to achieve fertilization than matings between unlike homozygotes, and that heterozygous embryos are more likely to succeed *in utero* than homozygotes, will be difficult with cattle for practical reasons. A more amenable animal will have to be found in which β-globulin polymorphism exists, and in which the β-globulin effect on fertility is operating. All mammals so far examined have shown this polymorphism and it is unlikely that the effect on fertility found in dairy cattle is unique. Mice show the polymorphism (Ashton & Braden, 1961; Cohen, 1960; Shreffler, 1960), but it is a rather specialized kind in which all but one strain so far examined are homozygous for one allele and the remaining strain is homozygous for another allele. No naturally occurring heterozygotes have been found. β-globulin polymorphism has also been observed in other laboratory animals (Ashton, 1958b) and one of these species may prove suitable.

ACKNOWLEDGMENTS

None of these data could have been collected without the active and generous co-operation of individual members of the Milk Marketing Board of England and Wales, the Queensland Department of Agriculture and Stock, and the National Cattle Breeding Station, 'Belmont', Rockhampton, Queensland. Too numerous to mention individually we are glad to acknowledge their valuable help. We are indebted to Dr J. M. Rendel and Mr H. G. Turner for constructive criticism of the manuscript, and to C. Bloomfield for technical assistance.

REFERENCES

Ashton, G. C. (1958a) Genetics of β-globulin polymorphism in British cattle. *Nature, Lond.* **182**, 370.

Ashton, G. C. (1958b) *Polymorphism in the serum proteins of mammals.* Ph.D. Thesis, University of Liverpool.

Ashton, G. C. (1960) β-globulin polymorphism and economic factors in dairy cattle. *J. agric. Sci.* **54**, 321.

Ashton, G. C. (1961) β-globulin type and fertility in artificially bred dairy cattle. *J. Reprod. Fertil.* **2**, 117.

Ashton, G. C. & Braden, A. W. H. (1961) β-globulin polymorphism in laboratory mice. *Aust. J. biol. Sci.* **14**, 248.

Boyd, H. & Reed, H. C. B. (1961) Investigations into the incidence and causes of infertility in dairy cattle—Fertility variations. *Brit. vet. J.* **117**, 18.

Cohen, B. L. (1960) Genetics of plasma transferrins in the mouse. *Genet. Res.* **1**, 431.

Fallon, G. R. (1958a) Artificial insemination of dairy cattle in Queensland. *Proc. Aust. Soc. Anim. Prod.* **2**, 90.

Fallon, G. R. (1958b) Some aspects of oestrum in cattle, with reference to fertility in artificial insemination. *Qld. J. agric. Sci.* **15**, 25.

GAHNE, B., RENDEL, J. & VENGE, O. (1960) Inheritance of β-globulins in serum and milk from cattle. *Nature, Lond.* **186**, 907.

KENNEDY, J. F. & TURNER, H. G. (1959) *A project on genetics of adaptation in cattle.* Commonwealth Scientific and Industrial Research Organization, Report No. 8 (Ser. S.W.-3), Div. Anim. Hlth. Prod., Australia.

OGDEN, A. L. (1959) Biochemical polymorphism in farm animals. *Proc. roy. Soc. Med.* **52**, 955.

ROBINSON, T. J. (1957) *Pregnancy.* Progress in the Physiology of Farm Animals, vol. 3, ed. J. Hammond. Butterworths, London.

SHREFFLER, D. C. (1960) Genetic control of serum transferrin type in mice. *Proc. nat. Acad. Sci.* **46**, 1378.

[*Reprinted from the Journal of Agricultural Science*, Vol. 54, No. 3, 1960.]

PRINTED IN GREAT BRITAIN

β-GLOBULIN POLYMORPHISM AND ECONOMIC FACTORS IN DAIRY CATTLE

By G. C. ASHTON*

The Animal Health Trust, Farm Livestock Research Station, Stock, Essex

(With Plate 11)

It is well-recognized that the yield of milk and butterfat of an individual cow is influenced by both non-genetic and genetic factors. Some progress has been made in understanding the main non-genetic factors. However, the genetic control of both milk yield and butterfat production is complex. Estimates of the heritability of each have been reported, but little is known about the individual genes controlling them.

Recently a number of simply inherited biochemical polymorphic systems have been described in the dairy cow, viz. β-lactoglobulins of milk (Aschaffenburg & Drewry, 1955, 1957), α-lactoalbumins of milk (Blumberg & Tombs, 1958), haemoglobins (Bangham, 1957), serum β-globulins (Ashton, 1957, 1958*a*; Smithies & Hickman, 1958), thread-proteins of serum (Ashton, 1958*b*) and slow-alpha proteins of serum (Ashton, 1958*c*). The genetics of bovine blood groups is also being investigated in a number of laboratories (Rendel, 1958). Each of these genetic systems is almost certainly being studied for evidence of involvement in milk yield and butterfat production. This communication presents data suggesting that the β-globulin alleles affect milk yield at least.

Difficulty is usually experienced in assessing the relative milk yield of different genotypes owing to the predominant effects of feeding and management (Robertson & Rendel, 1954). Even within the same herd and year non-genetic factors are responsible for more than half the differences in milk yield between cows, and if different herds and years are included this rises to about nine-tenths. In suitable circumstances the genotypic value of a bull for milk yield can be assessed far more accurately from the milk yield of his daughters than the genotypic value of a cow from her own yield. Thus any effect of the β-globulin alleles on milk yield would be detected most readily by considering the distribution of genotypic values with respect to β-globulin types of bulls.

The genotypic value of a bull for milk yield may be estimated from the contemporary comparison (Johanson & Robertson, 1952; Robertson & Rendel, 1954; Milk Marketing Board, 1954). This is an estimate of the milk yield of a given bull's daughters relative to daughters of other bulls milking under similar conditions of age, place, season and so on.

Butterfat percentage usually has a higher heritability than milk yield because it is less subject to management and environmental effects (Rendel, Robertson, Asker, Khiskin & Ragab, 1957). In this study therefore the effect of β-globulin genotype on butterfat percentage was investigated in several herds and the results from each herd assessed separately and comparatively.

MATERIALS

Blood samples. Blood samples from bulls with established contemporary comparison values were provided by the veterinary officers or managers of the twenty-two Milk Marketing Board, two Government and four privately-owned Cattle Breeding Centres in England and Wales.

Blood samples were also obtained from four Ayrshire herds in different parts of Great Britain. In these herds each animal which had completed at least one lactation was bled. Forty-six cows picked at random from a large pedigree Friesian herd were also bled.

Contemporary comparison and butterfat values. The contemporary comparison values for the bulls at the Cattle Breeding Centres were provided by the Consulting Officer Service of the Milk Marketing Board, and by the veterinary officers and managers of the Government and privately-owned centres. Because the reliability of the information provided by the contemporary comparison will be dependent on the relationship between the number of daughters and the number of contemporaries in a given herd, and also on the total number of herds in which daughters are located, it is necessary to take into account the 'weight' which may be attached to the information. This is obtained from the expression: $W = (n_1 \times n_2)/(n_1 + n_2)$, where n_1 is the number of daughters by the bull and n_2 is the number of contemporaries in a given herd. This value is calculated for each herd, and the values summed to

* Present address: C.S.I.R.O. Cattle Research Laboratory, Rockhampton, Queensland, Australia.

give the weight W. If W is less than 20 the contemporary comparison estimate is considered unreliable. Bulls with contemporary comparison values based on weights, W, of less than 20 are not included in the Results section.

The amount of butterfat in the milk of individuals in the Ayrshire herds was determined by the milk-testing laboratories of the National Milk Recording Scheme, and for the Friesian herd by the dairy associated with the herd. Each figure is the mean of between six and eleven analyses during the individual's first completed lactation. Lactations of less than 200 or longer than 350 days were not included.

METHODS

Starch-gel electrophoresis. The β-globulin type of the serum sample was determined by starch-gel electrophoresis in phosphate buffer (Ashton, 1957) using a slight modification of the procedure developed by Smithies (1955). The apparatus employed was similar to that of Smithies, except that all four vessels were filled with electrolyte (42 g./l. anhydrous disodium hydrogen phosphate, adjusted to pH 7·6 with saturated potassium dihydrogen phosphate solution). Platinum and not silver/silver chloride electrodes were used.

The hydrolysed potato starch was prepared from Hopkin and Williams potato starch, code 8208, by incubating in a 250 ml. conical flask 50 g. starch with 100 ml. of acetone and 1·5 ml. of hydrochloric acid sp.gr. 1·18 for 1½ hr. at 38·5° C. Several such flasks were treated at one time, and the contents were adjusted to pH 6·5–7·0 with saturated sodium acetate and the hydrolysed starch filtered off on a Buchner funnel. After washing with distilled water the cake was washed with a few hundred ml. of acetone and dried in an air oven at 40–45° C. To minimize variability in the hydrolysed product not less than 5 kg. was prepared and thoroughly mixed before being used for preparing gels.

The starch-gels were prepared at a concentration of 15% (w/v) from the hydrolysed potato starch and a 1 in 50 dilution of electrolyte. The hot preparation was poured into Perspex trays 25 × 4 × 0·5 cm., covered and left to stand overnight. The following morning the serum samples were inserted in the gel on pieces of Whatman no. 100 filter-paper 0·5 × 1·3 cm., thus permitting three samples per gel. The centre sample was usually a β^{AE} standard to permit easy identification of the sample serum on each side. The electrophoresis was carried out for 5 hr. at an applied voltage of 250 V. d.c., and a current of 12–15 mA./4 cm. gel. After 15 min. the power was turned off and the filter-paper inserts removed; this minimized streakiness in the stained gels.

At the end of the electrophoresis the gels were sliced with a fine wire and each half stained for 5 min. in a 1% solution of water-soluble nigrosine (B.D.H. Ltd.) in methanol 75 parts, water 25 parts, glacial acetic acid 10 parts by volume. The stained gels were washed overnight in methanol 50 parts, water 50 parts, glacial acetic acid 10 parts, and the following morning transferred to distilled water for 'typing'.

Identification of the β-globulin type. Six β-globulin types are present in British breeds of cattle (Ashton, 1958*a*). These represent the three homozygotes and three heterozygotes of three alleles β^A, β^D, and β^E. Each allele gives rise to four zones in starch gel. The most rapidly migrating β-globulin zone (i.e. the one nearest the albumin zone, Ashton, 1957) is rather faint and not readily seen. The next slowest zone stains moderately intensely, and the two slowest zones stain more or less equally intensely. However, the relative position of the group of four zones controlled by each allele differs with the allele. The zones controlled by β^A migrate more rapidly than those controlled by β^D, which in turn migrate more rapidly than those controlled by β^E. Each of the three homozygous types (genotypes β^A/β^A, β^D/β^D, and β^E/β^E, phenotypes β^{AA}, β^{DD} and β^{EE}) appears as a group of four zones as described. The heterozygous genotypes β^A/β^D, β^A/β^E, and β^D/β^E (phenotypes β^{AD}, β^{AE}, and β^{DE}) appear as combinations of the groups of four zones from each allele. In the case of phenotype β^{AE} this is usually quite readily seen if three sera in the sequence β^{AA}, β^{AE}, β^{EE}, are run side-by-side on the one gel. In the case of phenotypes β^{AD} and β^{DE} some of the zones overlap. However, the relationship is made clear by running the heterozygous serum between the two appropriate homozygous sera. The six β-globulin types are illustrated in Pl. 11.

For easy identification it is convenient to run a central β^{AE} reference serum on each gel. However, experience with the technique soon leads to rapid and correct identification without this aid.

RESULTS

The distribution of contemporary comparison values of bulls with respect to β-globulin type

Table 1 shows the distribution of the contemporary comparisons of all A.I. bulls in this study with respect to β-globulin type. Because of the low frequency of β^E in British breeds (Ashton, 1958*a*), very few β^{AE} and β^{DE}, and no β^{EE} animals are included.

The mean value for the sample of A.I. bulls is about +25 gal., compared with +10 gal. for a random sample of 1028 bulls noted by the Milk Marketing Board (1956). This suggests that, on average, the A.I. bulls in the sample have a some-

what higher genotypic value for milk yield. This is an expected and desirable finding. However, within the sample of A.I. bulls, the distribution of the contemporary comparison values for the three types β^{AA}, β^{AD} and β^{DD} is noticeably different. Thus, 64 % of the β^{AA} bulls but only 33 % of the β^{DD} bulls are poorer than the mean of +25 gal. The β^{AD} bulls are intermediate, about equal numbers of bulls of this group being better or worse than the +25 gal. mean.

The contemporary comparison values for the β^{AA}, β^{AD}, and β^{DD} bulls were grouped in 10 gal. groups (Table 1). The mean value for each curve and its standard deviation, and the standard errors of the mean and of its standard deviation, were calculated (Table 2). The value of the β^{AA} mean (+12·2 gal.) differs significantly (t = 2·70, 67 D.F., $P < 0{\cdot}01$) from the β^{DD} mean (+38·2 gal.). The genetically intermediate β^{AD} bulls also give an intermediate mean contemporary comparison value (+26·8 gal.). One can conclude that in this group of 130 bulls, at least, the β-globulin genotype has had a significant effect on the contemporary comparison values of the bulls.

The data in Table 1 can be partitioned in two ways, with respect to breed, and with respect to origin. Table 3 shows the distribution of contemporary comparison values with respect to breed and β-globulin type. Although the majority of the data come from Friesians it is fairly clear that the same phenomenon is shown by the other breeds also.

Table 4 shows the distribution with respect to the origin of the bulls, i.e. Milk Marketing Board bulls or bulls from other Centres. Unfortunately the majority of the bulls fall into the first group and there are insufficient other bulls to draw conclusions.

Shape of the distribution curves given by the contemporary comparison values

In assessing the significance of the difference between the means by the t test it is assumed that the contemporary comparison values for each of

Table 1. *Distribution of contemporary comparison values in* 10 *gal. groups*

Contemporary comparison group	No. of bulls in each group*				
	A.I. bulls				Random sample
	All	β^{AA} bulls	β^{AD} bulls	β^{DD} bulls	
Below −130	—	—	—	—	1
−120 to −129	—	—	—	—	3
−110 to −119	—	—	—	—	4
−100 to −109	—	—	—	—	3
− 90 to − 99	1	1	—	—	8
− 80 to − 89	0	0	—	—	5
− 70 to − 79	2	1	1	—	11
− 60 to − 69	1	0	1	—	25
− 50 to − 59	1	1	0	—	32
− 40 to − 49	2	0	2	—	38
− 30 to − 39	1	1	0	—	58
− 20 to − 29	11	5	5	1	60
− 10 to − 19	9	5	3	1	68
0 to − 9	7	0	6	1	76
+ 10 to + 1	13	8	2	3	84
+ 20 to + 11	10	1	6	3	78
+ 30 to + 21	8	2	4	2	82
+ 40 to + 31	14	3	8	3	86
+ 50 to + 41	20	4	8	8	62
+ 60 to + 51	7	2	3	2	55
+ 70 to + 61	8	2	4	2	42
+ 80 to + 71	3	0	2	1	42
+ 90 to + 81	4	1	2	1	23
+100 to + 91	3	1	1	1	23
+110 to +101	3	1	1	1	16
+120 to +111	1	—	1	—	11
+130 to +121	0	—	0	—	10
+140 to +131	1	—	1	—	6
+150 to +141	—	—	—	—	4
+160 to +151	—	—	—	—	5
Above +160	—	—	—	—	7
Totals	130	39	61	30	1028

* In addition there were seven β^{AE} bulls with values of − 48, − 28, − 5, +9, +28, +72 and +145 gal., and four β^{DE} bulls with values of − 16, +30, +35 and +40 gal., in the sample of 141 A.I. bulls.

the three β-globulin groups are normally distributed. Usually one would not test such data for departure from normality as slight skewness would not affect dependent tests of significance appreciably. However, it is probable that if the three β-globulin curves comprise contemporary comparison values distributed normally around their individual means, then the combined data without respect to β-globulin type would not be normally distributed. Accordingly, each of the sets of values for the β^{AA}, β^{AD} and β^{DD} bulls was tested for departure from normality by calculating the third and fourth moments as described by Fisher (1928). From the third moment γ_1 was calculated, giving a measure of the symmetry of the curve, and from the fourth moment γ_2 which gives a measure of departure in shape from the normal distribution curve. For this test the contemporary comparison values were distributed in 10 gal. groups. The loss of information due to grouping is less than 1% if the group interval does not exceed one-quarter of the standard deviation, in this instance about

Table 2. *Means, standard deviations, and standard errors of contemporary comparison data in Table* 1, *in gallons*

	Mean	S.E. of mean	Standard deviation	S.E. of standard deviation
β^{AA} bulls	+12·2	±6·92	±43·2	±4·90
β^{AD} bulls	+26·8	±5·37	±42·0	±4·71
β^{DD} bulls	+38·2	±5·51	±30·2	±3·90

Table 3. *Distribution of β-globulin types with respect to breed and contemporary comparisons in gallons*

β-Globulin type	Breed	No. of bulls with contemporary comparisons of +25 and below	No. of bulls with contemporary comparisons of +26 and above	Mean contemporary comparison
β^{AA}	Friesian	11	8	+12·8
β^{AD}	Friesian	14	19	+30·7
β^{DD}	Friesian	4	9	+41·2
β^{AA}	Shorthorn	5	6	+15·1
β^{AD}	Shorthorn	5	3	+24·1
β^{DD}	Shorthorn	1	2	+33·4
β^{AA}	Ayrshire	2	0	+16·5
β^{AD}	Ayrshire	4	5	+26·0
β^{DD}	Ayrshire	3	5	+32·5
β^{AA}	Channel Isle	7	0	− 7·3
β^{AD}	Channel Isle	6	5	+18·3
β^{DD}	Channel Isle	2	4	+38·2

Table 4. *Distribution of β-globulin types and contemporary comparisons with regard to the origin of the bulls*

β-Globulin type	Origin	No. of bulls with contemporary comparisons of +25 and below	No. of bulls with contemporary comparisons of +26 and above	Mean contemporary comparison
β^{AA}	M.M.B. centres	22	10	+ 7·3
β^{AD}	M.M.B. centres	20	28	+26·9
β^{DD}	M.M.B. centres	6	18	+38·8

β-Globulin type	Origin	No. of bulls with contemporary comparisons of +15 and below	No. of bulls with contemporary comparisons of +16 and above	Mean contemporary comparison
β^{AA}	Other centres	2	5	+34·7
β^{AD}	Other centres	4	9	+26·5
β^{DD}	Other centres	2	4	+32·2

± 40 gal. The values for γ_1 and γ_2 calculated from these data are shown in Table 5, together with the standard errors of γ_1 and γ_2 for a normal distribution. It is clear from Table 5 that neither the β^{AA}, β^{AD} nor β^{DD} distributions depart from normal.

The values γ_1 and γ_2 were next calculated for the 130 bulls as a whole without reference to β-globulin type. Table 5 shows that both γ_1 and γ_2 exceed their standard errors, suggesting that the curve is asymmetrical, although the values do not reach 1·96 times their respective standard errors and cannot be considered statistically significant. The distribution curves for the three β-globulin groups comprising the same data were then superimposed at their individual means. The distribution of the superimposed contemporary comparison values around this arbitrary mean was checked for normality. The calculation gave

$$\gamma_1 = -0{\cdot}007 \pm 0{\cdot}215 \text{ and } \gamma_2 = 0{\cdot}148 \pm 0{\cdot}430,$$

i.e. the same data re-arranged give no evidence of departure from a normal distribution.

In addition to the data presented here the Milk Marketing Board (1956) has available contemporary comparison data for 1028 bulls, drawn at random with respect to β-globulin type, and as far as is known with respect to all other factors including breed. The data are shown in Table 1. From Table 5 it is evident that the derived values of γ_1 and γ_2 are greater than their standard errors. The value of γ_1 differs significantly from zero when compared with its standard error ($P < 0{\cdot}001$), while the value of γ_2 is approaching significance ($P \simeq 0{\cdot}1$). The curve therefore is markedly asymmetrical. It seems reasonable to infer from this that the effect of the β-globulin alleles on contemporary comparison will not be confined to the 130 bulls in the present study, but will be evidenced also in other groups picked at random, such as the 1028 bulls discussed above.

The shape of the skewed distribution curves given by the 130 A.I. and 1028 random sample bulls is similar judging from the signs of γ_1 and γ_2. The positive value of γ_1 in both cases indicated an asymmetrical distribution in which the value of the mean is greater than the median (Snedecor, 1946). For the sample of 1028 bulls the median is between 0 and +10 gal. and the mean +10·3 gal. For the 130 A.I. bulls the median is between +24 and +25 gal. and the mean +25·2 gal.

The positive values of γ_2 are somewhat surprising at first sight, indicating a leptokurtic rather than the expected platykurtic curve. However, it can be shown graphically that a markedly asymmetric leptokurtic-like curve would result from differential culling, provided culling was strongest on the very poor bulls but progressively weaker as the bulls' genotypic value improved. There is evidence for such culling because the mean contemporary comparison of the random sample of 1028 bulls is not zero but +10·3 gal., while the mean of the 130 A.I. bulls is +25·2 gal.

Distribution of butterfat percentages with respect to β-globulin type

The distributions of mean first lactation butterfat percentages for cows in four Ayrshire and one Friesian herd are shown in Table 6. The results do not show any significant difference between the mean percentages for the various β-globulin types in each herd. However, in three of the four Ayrshire herds the β^{AE} and β^{DE} individuals tend to have a higher average butterfat percentage than the other types.

The mean butterfat percentage given by the progeny of each of the A.I. bulls in the study was available. For the Friesian bulls the means were β^{AA} 3·66%, β^{AD} 3·67%, β^{DD} 3·67%, and for two only β^{AE} bulls 3·67%. There were too few Ayrshire bulls to warrant any conclusions, and β^{E} was not represented amongst the Shorthorn or Channel Island bulls.

Further information will be required to establish whether these results reflect a genuine superiority of heterozygous β^{E} types with respect to mean butterfat percentage.

DISCUSSION

Magnitude of the effect of the β-globulin locus on milk yield

The difference between the mean contemporary comparison values for the β^{AA} and β^{DD} bulls will give a measure of the average effect on milk yield caused by completely replacing the allele β^{A} with β^{D} at the β-globulin locus.

Table 5. *Test of normality of distribution of contemporary comparison data in Table* 1

	γ_1	S.E. of γ_1	γ_2	S.E. of γ_2	Distribution
All bulls	0·236	±0·215	0·447	±0·430	Not normal
β^{AA} bulls	0·050	±0·391	−0·034	±0·748	Normal
β^{AD} bulls	0·037	±0·314	0·067	±0·627	Normal
β^{DD} bulls	0·035	±0·447	−0·232	±0·894	Normal
Random sample	0·257	±0·076	0·238	±0·153	Not normal

Table 6. *Distribution of first lactation butterfat percentages with respect to β-globulin type*

	β-Globulin type					
	β^{AA}	β^{AD}	β^{DD}	β^{AE}	β^{DE}	β^{EE}
Ayrshire herd 1						
No. of animals	1	9	12	3	3	2
Mean %	(3·80)	3·74	3·83	4·08	4·09	3·99
Ayrshire herd 2						
No. of animals	0	7	6	1	12	2
Mean %	—	4·03	4·11	(4·67)	4·17	3·85
Ayrshire herd 3						
No. of animals	1	6	2	0	2	0
Mean %	(3·61)	3·98	3·96	—	4·23	—
Ayrshire herd 4						
No. of animals	2	13	6	3	5	0
Mean %	3·73	4·00	3·87	4·17	3·93	—
Mean, % four Ayrshire herds	3·72	3·94	3·91	4·20	4·11	3·93
Friesian herd						
No. of animals	8	24	14	0	0	0
Mean %	3·69	3·74	3·87	—	—	—

However, the contemporary comparison values as determined take no account of the β-globulin type of the heifers. The daughters of β^{DD} bulls could be β^{DD}, β^{AD} or β^{DE}, the relative proportion of each type of offspring depending on the breed gene frequencies. For Friesian herds the great majority of offspring from β^{DD} bulls will be almost equally β^{AD} or β^{DD}, with very few β^{DE} types. In the same way the daughters from β^{AD} bulls will be β^{AA}, β^{AD}, β^{DD}, in the approximate ratio 1:2:1 with very few β^{AE} and β^{DE} types, while the daughters of β^{AA} bulls will be almost equally β^{AA} and β^{AD} with very few β^{AE}. It seems reasonable to infer that the mean contemporary comparison value of +12·2 gal. for the β^{AA} bulls would be less if the value had been determined by comparing the milk yields of the bull's β^{AA} daughters only with the average milk yield of his daughters' contemporaries. This could be termed for convenience the 'like-type contemporary comparison'. In the same way the like-type contemporary comparison for β^{DD} bulls could be reasonably expected to be greater than the observed mean of +38·2 gal. Little difference in the mean like-type and ordinary contemporary comparison value of β^{AD} bulls (+26·8 gal.) would be expected, however. Very approximately the like-type contemporary comparison value for β^{DD} bulls might be expected to be $\{26{\cdot}8+2(38{\cdot}2-26{\cdot}8)\} = +49{\cdot}6$ gal., while the same value for β^{AA} bulls might be about $\{26{\cdot}8-2(26{\cdot}8-12{\cdot}2)\} = -2{\cdot}4$ gal. The interpretation of this would be that a random sample of β^{AA} cows mated at random to the β^{AA} bulls in this study would give heifers with mean milk yield 52 gal. less than the heifers produced by comparable β^{DD} matings. It would be interesting to know if this average genetic superiority of +52 gal. of the β^{D} allele over the β^{A} allele is the same for all breeds.

It will be remembered that the shape of the distribution curves for the contemporary comparisons of both the 130 A.I. bulls and the 1028 random sample bulls suggested that differential culling had occurred. If this is so β^{AA} bulls would be more likely to be discarded than β^{DD} bulls. Although the means for all the groups would have an enhanced value as a result of such culling, the β^{AA} mean would be most affected, and the β^{DD} mean the least. The net result would be to narrow the margin between the average contemporary comparison of the β^{AA} and β^{DD} bulls, and hence lead to an underestimate of the effect of the β-globulin locus on milk yield.

The total genetic variation in milk yield in the major milking breeds can be assessed from the range of contemporary comparisons, i.e. about −130 gal. to +160 gal., say 300 gal. The β-globulin locus in British breeds of cattle, therefore, is responsible for about one-sixth, or perhaps more, of the total genetic variation.

β-Globulin type as an aid in the selection of superior bulls for milk yield

With the rapid expansion in artificial breeding of cattle since the war, considerable effort has been directed towards the recognition of superior bulls for use at Cattle Breeding Centres. Until fairly recently the average milk yield of cows bred artificially was not superior to the average of those bred naturally using within-herd comparisons (Robertson & Rendel, 1954). This was due to the methods of bull selection used, which did not differ

in principle from the largely subjective methods used by the average breeder. Following the development of the contemporary comparison method of assessment a rational system for bull selection has become available to the A.I. movement. However, a somewhat serious drawback to the rapid expansion of this principle of selection is that a bull is at least 6 years old before his first contemporary comparison estimate is known. Any method of post-natal selection which increases the chances of rearing bulls of higher-than-average contemporary comparison is therefore potentially valuable.

The ability of an individual bull to produce better than average milk yielding daughters cannot be forecast from his β-globulin type. Thus, individual β^{AA} bulls may be superior to individual β^{AD} or β^{DD} bulls. This is because the inherited level of milk production in an individual heifer is governed by many sets of genes, the β-globulin alleles presumably being one. In an unselected heifer population the alleles of all these genes could be expected to be distributed at random (omitting considerations such as linkage). Examination of milk yields in terms of only one of these genes should result in a series of normal distribution curves, one for each allele. The difference between the mean of each curve will represent the effect of each allele, and the spread of milk yields around each mean the effect of all the other genes concerned with milk yield. The standard deviation of the curves should not be different, therefore, as the net effect of all the other genes should be the same in each case. In fact, the standard deviations for the β-globulin curves are not significantly different (Table 2).

However, the findings reported in this communication might be of practical importance to the animal breeder concerned with milk yield in two ways.

First, providing the sample of bull calves is drawn at random, one can calculate from the properties of a normal distribution curve the percentage of animals which might eventually be expected to have contemporary comparisons better than a given value. Table 7 shows percentages for contemporary comparisons of +75 and +100 gal., found by referring the value x (see footnote to Table 7) to Fisher & Yates (1953) table IX. When an early selection of bull calves has to be made because of management considerations, the chance of rearing superior bulls would appear to be increased by selecting only β^{DD} animals.

Secondly, homogeneous herds of β^{DD} cows and heifers sired by the best β^{DD} bulls should lead to improved mean herd milk yields.

There would be other advantages in the extended use of β^{DD} bulls to herds composed only of β^{DD} cows. It has been shown elsewhere (Ashton, 1959) that the number of female offspring from matings between like homozygotes is greater than that from matings between homozygotes and heterozygotes when the two alleles β^A and β^D only are involved.

β-globulin type and butterfat percentage

The data on the relationship between butterfat percentage and β-globulin type are insufficient to permit definite conclusions to be drawn. However, there is an indication that β^{AE} and β^{DE} heifers may give, on average, a higher butterfat percentage than β^{AA}, β^{AD}, or β^{DD} heifers. If substantiated this might give a clue to the reason for the retention of β^E in British breeds, notwithstanding its disadvantage to the individual (Ashton, 1958, 1959). This disadvantage (apparent incompatability between β^E mother and her offspring) might rule against selection of β^E individuals as a means of increasing herd butterfat production.

SUMMARY

1. The serum β-globulin phenotype of 141 bulls located at Cattle Breeding Centres in England and Wales was determined by starch-gel electrophoresis.

2. The distribution of the contemporary comparison values of these bulls with respect to β-globulin type was investigated. The great majority of the bulls were β^{AA}, β^{AD}, or β^{DD} with very few β^{AE} and β^{DE} and no β^{EE} types.

3. The contemporary comparison values for 130 bulls of the three types β^{AA}, β^{AD} and β^{DD} each fell into a normal distribution. The mean and its

Table 7. *Expected percentage of bulls with contemporary comparisons of +75 or more, or +100 or more*

	Contemporary comparison values of					
	+75 or more			+100 or more		
	β^{AA}	β^{AD}	β^{DD}	β^{AA}	β^{AD}	β^{DD}
Value of X*	1·506	1·156	0·882	2·106	1·755	1·482
Expected percentage†	5·6	12·5	18·9	1·76	3·95	7·95

* $X = \dfrac{75 \text{ (or } 100) - \text{mean contemporary comparison}}{\text{average standard deviation of mean (41·7)}}$.

† Calculated from table IX of Fisher & Yates's tables (1953).

standard error and the standard deviation of the mean and its standard error were calculated for each of the three curves. The mean contemporary comparison value for the β^{AA} bulls was $+12{\cdot}2 \pm 5{\cdot}4$ gal., for the β^{AD} bulls $+26{\cdot}8 \pm 5{\cdot}4$ gal., and for the β^{DD} bulls $+38{\cdot}2 \pm 5{\cdot}5$ gal. The β^{AA} and β^{DD} means were significantly different ($P < 0{\cdot}01$).

4. The pooled contemporary comparison values of the β^{AA}, β^{AD} and β^{DD} bulls did not give a normal distribution curve, neither did the contemporary comparison values for a sample of 1028 bulls quoted by the Milk Marketing Board. However, when the contemporary comparison values of the β^{AA}, β^{AD} and β^{DD} groups were superimposed around the same mean, the ensuing distribution was normal.

5. It is concluded that the β-globulin locus is concerned in the genetic control of milk yield. The estimated mean genetic value of β^{D} over β^{A} is approximately +50 gal. This is about one-sixth of the total genetic variation in milk yield in the major milk breeds.

6. The distribution of butterfat percentages with respect to β-globulin phenotype in four Ayrshire and one Friesian herds was investigated. No difference between the mean first lactation butterfat percentage of the β^{AA}, β^{AD}, and β^{DD} cows in each herd was found. There was an indication (not significant) that the mean butterfat percentage of the β^{AE} and β^{DE} cows in three of the four Ayrshire herds studied was higher than the mean of the other groups.

I wish to thank the Chief Veterinary Officer and Chief Consulting Officer of the Milk Marketing Board, and the managers and veterinary officers of the Government and privately-owned Cattle Breeding Centres for generous co-operation in many phases of this and other similar work. Without their help it would not have been possible to obtain adequate numbers of representative blood samples nor to correlate β-globulin data with other factors.

Also, I wish to thank Dr J. M. Rendel, Dr A. Robertson, and Mr H. G. Turner for their comments on the draft typescript.

REFERENCES

Aschaffenburg, R. & Drewry, J. (1955). *Nature, Lond.*, **176**, 218.

Aschaffenburg, R. & Drewry, J. (1957). *Nature, Lond.*, **180**, 376.

Ashton, G. C. (1957). *Nature, Lond.*, **180**, 917.

Ashton, G. C. (1958*a*). *Nature, Lond.*, **182**, 370.

Ashton, G. C. (1958*b*). *Nature, Lond.*, **182**, 65.

Ashton, G. C. (1958*c*). *Nature, Lond.*, **182**, 193.

Ashton, G. C. (1959). *Nature, Lond.*, **183**, 404.

Bangham, A. D. (1957). *Nature, Lond.*, **179**, 467.

Blumberg, B. S. & Tombs, M. P. (1958). *Nature, Lond.*, **181**, 683.

Fisher, R. A. (1928). *Statistical Methods for Research Workers*, 2nd ed. Edinburgh: Oliver and Boyd.

Fisher, R. A. & Yates, F. (1953). *Statistical Tables for Biological, Agricultural and Medical Research*, 4th ed. Edinburgh: Oliver and Boyd.

Johanson & Robertson, A., I. (1952). *Brit. Soc. Anim. Prod. Proc.* p. 79.

Milk Marketing Board (1954). *Production Division Report*, No. 4, 16.

Milk Marketing Board (1956). *The Development and Use of the Contemporary Heifer Comparison in Interpreting Progeny Lactation Records. Internal Report*, no. X, 166.

Rendel, J. (1958). *Acta Agric. Scand.* **8**, 40.

Rendel, J. M., Robertson, A., Asker, A. A., Khiskin, S. S. & Ragab, M. T. (1957). *J. Agric. Sci.* **48**, 426.

Robertson, A. & Rendel, J. M. (1954). *J. Agric. Sci.* **44**, 184.

Smithies, O. (1955). *Biochem. J.* **61**, 629.

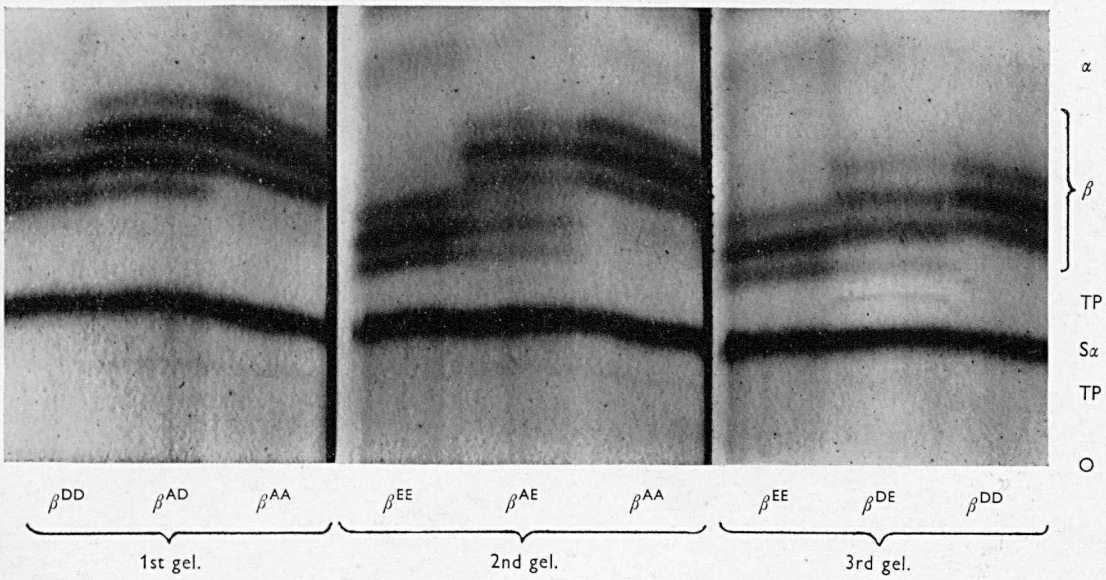
Smithies, O. & Hickman, C. G. (1958). *Genetics*, **43**, 374.

Snedecor, G. W. (1946). *Statistical Methods*, 4th ed. Ames, Iowa: Iowa State College Press.

EXPLANATION OF PLATE 11

β-Globulin phenotypes in British cattle breeds. The photograph shows three gels each carrying three serum samples. α = part of the α-globulin complex; β = β-globulins; TP = thread-proteins; $S\alpha$ = slow alpha globulins; 0 = original point of insertion of serum sample.

(*Received* 13 *July* 1959)


α
β
TP
Sα
TP
O
β^{DD} β^{AD} β^{AA} β^{EE} β^{AE} β^{AA} β^{EE} β^{DE} β^{DD}
1st gel.
2nd gel.
3rd gel.

(*Facing p.* 328)

Commonwealth of Australia
COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION
J. Agric. Sci. (1964), **62**, 27
Printed in Great Britain

(9)

Transferrin (β-globulin) type and milk and butterfat production in dairy cows

By G. C. ASHTON, G. R. FALLON and D. N. SUTHERLAND

Cattle Research Laboratory, C.S.I.R.O., Rockhampton, Queensland; Veterinary School, University of Queensland; and Department of Agriculture and Stock, Brisbane, Queensland

(*Received* 8 *February* 1962)

It was reported by Ashton (1960) that the transferrin (β-globulin) locus in cattle is concerned in the genetic control of milk yield. From a study of the distribution of contemporary comparisons of 130 bulls with regard to transferrin type it was found that the mean value for the *TfDD* bulls in the sample was +38·2 gallons, for the *TfAD* bulls +26·8 gallons, and for the *TfAA* bulls +12·2 gallons. It was suggested that the difference in milk yield between an unselected group of *TfAA* cows and a similar group of *TfDD* cows of the same breed therefore might be about 50 gallons. Accordingly the relationship between milk yield and transferrin type was investigated in thirteen herds of Jersey cattle and twenty-four herds of Australian Illawarra Shorthorn cattle located in South East Queensland.

The results confirm that the transferrin locus does influence milk yield in cows, to about the extent forecast from the contemporary comparison data.

MATERIALS AND METHODS

Blood samples. Blood samples were obtained as far as practicable from all the milking cows and heifers in thirteen herds in the Nambour region of Queensland, and twenty-four herds in the Kingaroy region of Queensland. The Nambour herds were nominally Jersey and the Kingaroy herds nominally Australian Illawarra Shorthorn. The herds can be regarded as a random sample of herd-recorded herds in their respective districts. They provided the data for the transferrin fertility studies reported elsewhere (Ashton, 1961; Ashton & Fallon, 1962). These herds are probably of a higher standard of management than non-recorded herds.

The transferrin type was determined by starch-gel electrophoresis (Smithies, 1955; Ashton, 1960).

Nomenclature of transferrin types. Giblett, Hickman & Smithies (1959) have demonstrated that the polymorphic β-globulins bind iron and are identical with the previously recognized iron-binding protein termed variously transferrin or siderophilin. The locus symbol β has therefore been replaced by *Tf* in humans, and this nomenclature has been recommended for adoption in other species where this polymorphism occurs. The locus symbols β and *Tf* in cattle are therefore synonymous, β having priority, but *Tf* being more definitive.

Three transferrin alleles are present in most British breeds of cattle, viz. *TfA*, *TfD*, and *TfE*, giving six genotypes *TfA/TfA*, *TfA/TfD*, *TfD/TfD*, *TfA/TfE*, *TfD/TfE*, and *TfE/TfE* (Ashton, 1958; Smithies & Hickman, 1958). Each genotype gives an individually recognizable phenotype. For convenience, the locus symbol may be dropped, and the types referred to as *A/A*, *A/D*, and so on.

Milk and butterfat records. In all herds, recording of milk yield and butterfat test was carried out under the Grade Herd Recording scheme of the Queensland Department of Agriculture and Stock. Production of individual cows in milk was recorded on one day of each month.

Difficulty is always experienced in assessing milk yield data for evidence of genetic effects owing to the predominant effects of feeding and management (Robertson & Rendel, 1954). Within the same herd and year non-genetic factors are responsible for more than half the differences in milk yield between cows. If the comparison is made between different herds, and in different years, non-genetic variation may account for nine-tenths of the total. To reduce this variation as much as possible, only lactations starting in the 1958–59 season were included in the analysis of the Australian Illawarra Shorthorn data, and only those starting in the 1959–60 season in the analysis of the Jersey data. The lactation was included irrespective of whether it was a first or repeat lactation, although it is known that milk yield is influenced by lactation number.

Abnormally short or long lactations might influence the comparison unduly. Accordingly an arbitrary restriction of lactation lengths included in the data was imposed. For each herd the shortest and longest lactations included did not differ by more than 90 days. The actual range of lactation lengths covered depended on the mean lactation length for the herd, and was so chosen that the largest number of animals from that herd was

included. The ranges used were 180–270, 210–300, 240–330, and 270–360 days. The range for each herd is shown in Tables 1 and 2.

Analysis of data. The data were analysed by a least squares method using an additive model, assuming no herd × genotype interaction, i.e.

$$y_{ijk} = \mu + h_i + g_j + l_k$$

where $i = 1, 2, \ldots,$ number of herds,
$j = 1, 2, \ldots,$ number of genotypes,
$k = 1, 2, \ldots,$ number of cows,

such that $\sum_i h_i = 0$ and $\sum_j g_j = 0$,

it being assumed that the error term (l_k) was normally and independently distributed with unknown variance.

The analyses were carried out using the multiple linear regression programme for the G.E.C. 225 computer (General Electric Computer Dept., Phoenix, Arizona, U.S.A., June 1962). To fit the above model independent variables were generated according to the following table:

	Herd or genotype					
	1	2	3	4	...	a
Variable x_1	0	1	0	0		0
x_2	0	0	1	0		0
x_3	0	0	0	0		0
⋮						
x_{a-1}	0	0	0	0		1

The capacity of this computer is twenty-seven variables. Accordingly, the Jersey data were analysed completely (thirteen herds, three genotypes, and four other variables, namely milk yield, lactation length, fat yield, and fat percentage). This was not possible with the A.I.S. data (twenty-four herds, six genotypes, and the four other variables). For these data the analysis was carried out in parts:

(*a*) Three herds were omitted, viz. K 2, K 6, and K 13, and the association between the three transferrin types *A/A*, *A/D* and *D/D*, and milk yield and butterfat percentage computed.

The herds eliminated were those having the smallest total number of cows of these three genotypes.

(*b*) The analysis was repeated for the same herds and genotypes, for lactation length and butterfat yield.

(*c*) Three further herds were omitted, viz. K 5, K 11 and K 24, and the association between the six possible genotypes and milk yield and butterfat percentage computed.

The further herds eliminated were those not having *TfE* represented.

(*d*) Analysis (*c*) was repeated for lactation length and butterfat yield.

RESULTS

A. *Jersey data*

Only two transferrin alleles *TfA* and *TfD*, and three types *A/A*, *A/D*, and *D/D* occur in Jersey cattle. Table 1 shows the number of each of the three genotypes in each herd. The three transferrin types were represented in each herd, except in N 2 where *A/A* was absent. In order to allow an appraisal of the data Table 1 gives the genotype means within each herd for lactation length, milk yield in lb., butterfat yield in lb., and butterfat percentage. It will be appreciated, however, that the statistical analysis of the data used the individual values for each cow, and not the means shown in Table 1.

Table 3 shows the results of the least-squares multiple regression analysis in terms of differences between genotypes relative to type *A/A*, and the standard error of that difference. From Table 3 it may be seen that *D/D* cows gave 43·5 gallons of milk more than the *A/A* cows in the sample ($P < 0{\cdot}05$), 26·4 lb. of fat ($P < 0{\cdot}01$) and lactated for 19·7 days longer ($P < 0{\cdot}001$). Butterfat percentage did not differ significantly between these two genotypes.

A/D cows did not differ from *A/A* cows in milk yield, fat yield, or lactation length but *A/D* cows had 0·07 % less butterfat than *A/A* cows ($P < 0{\cdot}05$).

B. *Illawarra Shorthorn TfA and TfD data*

The means for the A.I.S. data are given in Tables 2*a* and *b*, although as in the case of the Jersey data the values for individual cows were used in the computations.

Table 4 shows the results of the analysis for the three genotypes *A/A*, *A/D*, and *D/D* in twenty-one of the twenty-four herds. More than 85 % of the cows in these herds were represented in the analysis. From this table it may be seen that compared with the *A/A* cows the *D/D* cows gave 46·3 gallons more milk ($P < 0{\cdot}01$), and lactated for 10·3 days longer ($P < 0{\cdot}05$). There were no significant differences between the two genotypes with respect to fat yield or butterfat percentage. In this analysis *A/D* cows were superior to *A/A* cows in milk yield (36·3 gallons, $P < 0{\cdot}05$), and lactated longer (11·8 days, $P < 0{\cdot}01$). No difference in fat yield or butterfat percentage was found.

C. *Illawarra Shorthorn data, all genotypes*

The results of the analysis for eighteen herds and six genotypes are shown in Table 5. Again, *D/D* cows are superior to *A/A* cows in milk yield (42·2 gallons, $P < 0{\cdot}05$), and lactate longer (9·5 days, $P < 0{\cdot}05$). These values from eighteen herds are very similar to those obtained from the twenty-one herds (Table 4). This also held for the *A/D–A/A*

Table 1. *Mean values for the different transferrin types in thirteen herds of Jersey cows for lactation length, milk yield, butterfat yield and butterfat percentage*

Herd no.	Range of lactation lengths included (days)	No. of each type in herd			Mean lactation length (days)			Mean milk yield (lb.)			Mean butterfat yield (lb.)			Mean % butterfat		
		A/A	*A/D*	*D/D*	*A/A*	*A/D*	*D/D*	*A/A*	*A/D*	*D/D*	*A/A*	*A/D*	*D/D*	*A/A*	*A/D*	*D/D*
N 1	270–360	5	4	1	294·0	300·0	300·0	4848	4496	4920	241·0	247·6	224·1	4·98	5·50	4·50
N 2	240–330	0	5	6	—	270·0	280·0	—	3741	3655	—	178·4	183·1	—	4·84	4·33
N 3	240–330	6	16	4	275·0	268·1	285·0	3463	3439	3450	168·9	164·9	166·9	4·92	4·85	4·85
N 4	240–330	4	9	2	262·5	283·3	300·0	3160	3764	4005	150·8	188·1	198·6	4·88	4·97	4·95
N 5	240–330	1	5	5	270·0	270·0	294·0	3695	4273	4669	181·0	225·0	258·4	4·90	5·30	5·58
N 6	270–360	8	12	1	300·0	310·0	330·0	6833	6441	7365	326·6	318·8	396·9	4·74	4·98	5·40
N 7	240–330	2	13	3	240·0	276·9	390·0	3225	3663	3935	191·1	184·6	197·6	5·85	5·05	5·10
N 8	240–330	10	6	1	261·0	290·0	300·0	3311	3624	5115	165·3	182·3	231·6	5·02	5·08	4·50
N 9	240–330	4	5	2	285·0	300·0	300·0	4589	4254	3925	201·7	197·3	170·3	4·50	4·66	4·35
N 10	240–330	14	12	4	278·6	277·5	292·5	4141	3787	4008	190·9	182·3	184·1	4·63	4·78	4·65
N 11	240–330	3	12	8	250·0	272·5	251·3	1943	2775	2870	92·8	129·5	124·7	4·87	4·67	4·40
N 12	240–330	6	10	6	280·0	297·0	290·0	3622	4122	4533	175·2	206·1	242·4	4·88	5·03	5·35
N 13	240–330	3	5	2	250·0	252·0	300·0	3615	3482	4035	165·7	175·6	203·4	4·63	5·00	5·10

differences, although only lactation length differed significantly in the reduced sample.

The main reason for this analysis was to assess the effect of the *TfE* allele on milk yield. Unfortunately the number of *E/E* animals was very small and the standard errors consequently very large. The difference of 51·0 gallons milk between *A/A* and *E/E* cows is not significant, therefore, and it is not possible to determine whether *E/E* cows are in fact superior to *A/A* cows from this sample. A larger population of cows with a higher frequency of *TfE* would be necessary to determine this.

The milk yields of the more numerous *A/E* and *D/E* cows did not differ significantly from the *A/A* cows although *A/E* cows had significantly longer lactations. No effect of transferrin type on butterfat yield or percentage was found.

D. *Combined Jersey and Illawarra Shorthorn data*

The combined genotype differences for the *A/A*, *A/D* and *D/D* types are shown in Table 6. The effect of this pooling is to increase the significance of the *A/A* and *D/D* milk yield comparison and establish its value as $45{\cdot}0 \pm 16{\cdot}2$ gallons. The lactation length difference emerges as $13{\cdot}9 \pm 4{\cdot}5$ days.

DISCUSSION

Milk yield. It was forecast from the distribution of contemporary comparisons of bulls of known genotype (Ashton, 1960) that the milk yield of a random sample of cows would be affected by transferrin type. The results presented here, from two unrelated dairy breeds in different regions of Queensland are in agreement with this forecast. Both the distribution of bulls' contemporary comparisons and the milk yields of cows of both breeds show that *D/D* types are superior, *A/D* intermediate and *A/A* poorest.

The difference in mean contemporary comparison between *A/A* and *D/D* bulls was found to be 26·0 gallons. Because homozygous bulls will produce approximately half homozygous and half heterozygous daughters in British breeds of dairy cattle it was suggested that the effect on milk yield would be about twice the observed difference in contemporary comparison, i.e. about 52 gallons. The observed differences in mean milk yield between *A/A* and *D/D* Jersey cows (43·5 gallons) and Illawarra Shorthorn cows (46·3 gallons) are fairly close to the difference postulated from the contemporary comparisons.

There is a suggestion that the milk yield of the rather rare *E/E* cows may be as good as that of the *D/D* cows. Because of the large standard error resulting from the small number of comparisons available it is not possible to determine whether this is so from the present data. There was no

Table 2*a*. *Mean values for the different transferrin types in twenty-four herds of Australian Illawarra Shorthorn cows for lactation length and milk yield*

Herd no.	Range of lactation lengths included (days)	No. of each type in herd						Mean lactation length (days)						Mean milk yield (lb.)					
		A/A	*A/D*	*D/D*	*A/E*	*D/E*	*E/E*	*A/A*	*A/D*	*D/D*	*A/E*	*D/E*	*E/E*	*A/A*	*A/D*	*D/D*	*A/E*	*D/E*	*E/E*
K 1	240–330	4	15	5	—	2	—	270·0	286·0	282·0	—	285·0	—	4676	6215	5949	—	3885	—
K 2	210–300	—	5	2	1	—	—	—	222·0	270·0	210·0	—	—	—	5403	6965	4200	—	—
K 3	240–330	3	7	1	1	1	—	280·0	252·9	300·0	300·0	270·0	—	3488	4955	5720	2355	3780	—
K 4	240–330	—	3	7	—	8	1	—	300·0	265·7	—	266·3	300·0	—	5580	5254	—	6186	6690
K 5	240–330	7	12	4	—	—	—	270·0	272·5	262·5	—	—	—	5144	4120	5055	—	—	—
K 6	210–270	—	—	8	—	6	—	—	—	240·0	—	235·0	—	—	—	5843	—	5195	—
K 7	240–330	1	8	8	1	—	—	240·0	281·3	277·5	240·0	—	—	4635	4814	4915	4945	—	—
K 8	240–330	1	5	5	4	3	1	270·0	294·0	282·0	285·0	270·0	300·0	7050	7230	7317	6409	4800	6240
K 9	240–330	2	10	11	1	3	—	240·0	273·0	259·1	300·0	240·0	—	4553	5716	6076	5040	6420	—
K 10	240–330	—	4	11	1	2	—	—	270·0	264·5	270·0	255·0	—	—	4728	5242	3540	4610	—
K 11	180–270	2	10	6	—	—	—	195·0	210·0	200·0	—	—	—	3180	3764	4085	—	—	—
K 12	210–300	3	10	12	2	3	1	240·0	234·0	227·5	270·0	240·0	210·0	2990	3966	3657	3345	3565	3090
K 13	210–300	—	6	8	—	—	—	—	240·0	258·8	—	—	—	—	3203	3534	—	—	—
K 14	180–270	2	12	5	2	2	2	210·0	220·0	222·0	225·0	195·0	240·0	4620	4736	4242	3750	4035	4470
K 15	210–300	1	3	3	—	—	1	210·0	240·0	240·0	—	—	210·0	3825	3811	3712	—	—	4549
K 16	210–300	2	8	2	1	3	—	255·0	266·3	300·0	270·0	280·0	—	6148	4609	6105	2985	7237	—
K 17	180–270	2	7	9	1	1	—	210·0	240·0	226·7	300·0	270·0	—	5610	5423	5312	7935	4200	—
K 18	210–300	1	8	10	—	1	—	240·0	243·8	255·0	—	240·0	—	4335	3998	3888	—	3960	—
K 19	240–330	—	11	6	—	3	—	—	261·8	265·0	—	280·0	—	—	5606	5237	—	6285	—
K 20	180–270	—	13	6	—	1	—	—	251·4	220·0	—	240·0	—	—	3225	3515	—	2985	—
K 21	210–300	1	5	7	—	2	—	240·0	240·0	231·4	—	210·0	—	3990	4426	4704	—	4270	—
K 22	210–300	2	7	2	1	—	—	255·0	248·6	225·0	240·0	—	—	4605	5282	4770	5085	—	—
K 23	210–300	4	7	5	—	—	1	217·5	270·0	258·0	—	—	240·0	2511	3521	3368	—	—	4005
K 24	240–330	—	7	8	—	—	—	—	265·7	277·5	—	—	—	—	6891	6953	—	—	—

Table 2*b*. *Continuation of Table 2a, for mean butterfat yield and butterfat percentage*

Herd no.	Mean butterfat yield (lb.)						Mean % butterfat					
	A/A	*A/D*	*D/D*	*A/E*	*D/E*	*E/E*	*A/A*	*A/D*	*D/D*	*A/E*	*D/E*	*E/E*
K1	177·9	243·8	227·3	—	152·4	—	3·80	3·92	3·82	—	3·92	—
K2	—	195·3	233·9	121·2	—	—	—	3·61	3·36	2·89	—	—
K3	119·0	194·3	219·0	74·1	155·7	—	3·41	3·92	3·83	3·15	4·12	—
K4	—	200·7	185·1	—	217·3	232·8	—	3·60	3·52	—	3·51	3·48
K5	179·8	162·6	179·6	—	—	—	3·50	3·95	3·55	—	—	—
K6	—	—	219·2	—	199·5	—	—	—	3·75	—	3·84	—
K7	180·9	192·7	198·7	198·8	—	—	3·90	4·00	4·04	4·02	—	—
K8	249·6	239·6	250·4	229·6	144·2	219·9	3·54	3·31	3·42	3·58	3·00	3·52
K9	183·9	210·3	220·5	195·3	230·8	—	4·04	3·68	3·63	3·88	3·60	—
K10	—	159·9	202·3	120·3	152·7	—	—	3·38	3·86	3·40	3·31	—
K11	121·5	161·3	175·0	—	—	—	3·82	4·29	4·28	—	—	—
K12	119·4	144·5	139·3	134·1	136·5	120·9	3·99	3·64	3·81	4·01	3·83	3·91
K13	—	119·3	137·3	—	—	—	—	3·72	3·89	—	—	—
K14	172·7	176·4	160·3	136·2	134·7	191·0	3·74	3·72	3·78	3·63	3·34	4·28
K15	138·6	135·1	119·3	—	—	167·9	3·62	3·55	3·21	—	—	3·69
K16	231·5	169·2	245·1	123·6	259·8	—	3·77	3·67	4·01	4·14	3·59	—
K17	261·3	200·6	196·0	271·8	134·4	—	4·66	3·70	3·69	3·43	3·20	—
K18	170·4	150·9	147·6	—	164·4	—	3·93	3·77	3·80	—	4·15	—
K19	—	223·0	201·0	—	253·9	—	—	3·98	3·84	—	4·04	—
K20	—	119·1	130·5	—	100·8	—	—	3·69	3·71	—	3·38	—
K21	156·9	175·3	179·2	—	164·7	—	3·93	3·96	3·81	—	3·86	—
K22	184·8	200·8	205·1	169·8	—	—	4·01	3·80	4·30	3·34	—	—
K23	103·2	130·1	120·2	—	—	159·3	4·11	3·69	3·57	—	—	3·98
K24	—	259·5	253·2	—	—	—	—	3·77	3·64	—	—	—

Table 3. *Showing the differences between genotypes A/A and D/D, and A/A and A/D in the Jersey cows with regard to lactation length, milk yield, butterfat yield, and butterfat percentage, A/A taken as base-line in each case. The standard errors of these differences are also shown*

Variable	Genotypes compared: D/D and A/A	Genotypes compared: A/D and A/A
Lactation length (days)	19·71 ± 7·26***	0·74 ± 2·42
Milk yield (lb)	434·5 ± 237·9*	−4·1 ± 79·2
Butterfat yield (lb.)	26·44 ± 12·10*	−3·56 ± 4·03
Butterfat (%)	0·021 ± 0·126	−0·071 ± 0·042*

* $P < 0·05$; *** $P < 0·001$.

Table 4. *Showing the difference between genotypes A/A and D/D, and A/A and A/D in twenty-one of the twenty-four herds of Australian Illawarra Shorthorn cows, with regard to lactation length, milk yield, butterfat yield, and butterfat percentage, A/A taken as base-line in each case. The standard errors of these differences are also shown*

Variable	Genotypes compared: D/D and A/A	Genotypes compared: A/D and A/A
Lactation length (days)	10·29 ± 5·67*	11·83 ± 5·39**
Milk yield (lb.)	463·0 ± 219·8**	363·4 ± 208·8*
Butterfat yield (lb.)	−0·65 ± 10·26	−5·55 ± 9·74
Butterfat (%)	0·001 ± 0·080	−0·002 ± 0·076

* $P < 0·05$; ** $P < 0·01$.

Table 5. *Showing the differences between genotype A/A, and the other five genotypes in eighteen of the twenty-four herds of Australian Illawarra Shorthorn cows, with regard to lactation length, milk yield, butterfat yield, and butterfat percentage, A/A taken as base-line in each case. The standard errors of these differences are also shown*

Variable	Genotypes compared: A/A and D/D	A/A and E/E	A/A and A/D	A/A and A/E	A/A and D/E
Lactation length (days)	9·52* ± 5·72	14·41 ± 12·40	12·65** ± 5·43	21·0** ± 9·08	5·77 ± 7·34
Milk yield (lb.)	421·5* ± 231·5	510·0 ± 501·4	352·5 ± 219·8	−247·7 ± 367·5	289·1 ± 297·0
Butterfat yield (lb.)	1·92 ± 12·77	14·6 ± 27·65	−3·99 ± 12·12	6·06 ± 20·27	10·05 ± 16·38
Butterfat (%)	0·019 ± 0·078	0·215 ± 0·170	−0·007 ± 0·074	−0·061 ± 0·124	−0·128 ± 0·101

* $P < 0·05$; ** $P < 0·01$.

Table 6. *Showing the differences between genotypes A/A and D/D, and A/A and A/D in the pooled Jersey and A.I.S. data, A/A taken as base-line in each case. The standard errors of these differences are also shown*

Variable	Genotypes compared: A/A and D/D	Genotypes compared: A/A and A/D
Lactation length (days)	13·86 ± 4·47**	2·59 ± 2·20
Milk yield (lb.)	449·7 ± 161·6**	42·1 ± 74·1
Butterfat yield (lb.)	11·50 ± 7·84	2·29 ± 3·72
Butterfat (%)	0·014 ± 0·067	−0·054 ± 0·037

** $P < 0·01$.

indication that the *A/E* or *D/E* cows gave as much milk as the *D/D* cows.

Lactation length. The mean difference between *A/A* and *D/D* cows in lactation length (Table 6) was 13·9 days. It is possible that the primary effect of the transferrin locus is on lactation length, and not on yield per given lactation day. However, the extra milk yield of *D/D* compared with *A/A* cows is approximately 45 gallons, i.e. about 3·2 gallons per extra day. This is in excess of the average daily yield of about 1½ gallons (Jersey) and 2 gallons (A.I.S.). The transferrin effect is not due simply to increased lactation length therefore.

Butterfat production. There is no consistent evidence that transferrin type affects butterfat percentage, although the data show that *A/D* Jersey cows have a significantly lower percentage ($P < 0·05$) than *A/A* cows.

The evidence for an effect on butterfat yield is conflicting. In the Jersey data D/D cows yielded 26·4 lb. more fat than A/A cows ($P < 0{\cdot}01$). In the A.I.S. data there was no difference between the fat yields of the A/A and D/D cows.

The need for more extensive data from these and other breeds of cattle is evident.

The extent of the transferrin effect on milk yield

The data included in the analyses above were derived from lactations in a given year, irrespective of the order number of the lactation. It would have been preferable to select only first lactation yields in the given year, and so reduce bias due to culling. In Queensland very few animals are culled as a result of first lactation production, most cows being kept at least for a second lactation. However, the herds represented had almost certainly culled some animals, and it is possible that the culled animals would have included a greater relative percentage of A/A cows than D/D cows. The difference between the two genotypes may be underestimated, therefore, in the same way that the difference between the A/A and D/D bulls contemporary comparisons were underestimated because of differential culling.

Assuming, however, that the difference between A/A and D/D cows, and hence the effect of replacing TfA by TfD at the transferrin locus, is 43·5 gallons for Jerseys and 46·3 gallons for A.I.S., then it is possible to relate this to the total genetic variation in milk yield and to estimate the proportion of the variation due to the transferrin locus.

In the Jersey population reported here the mean milk yield for the 225 cows was 400·4 gallons, and the phenotypic standard deviation was ± 87·34 gallons. The heritability of milk yield in this particular population is not known but an assumed value of 0·3 would seem reasonable (Johansson, 1950). The genetic variance would then be $87{\cdot}34^2 \times 0{\cdot}3 = 2288$ gal.^{2}. The gene frequencies in this population were $TfA = 0{\cdot}547$ and $TfD = 0{\cdot}453$. The population consisted therefore of:

29·9 % TfA/A cows with genetic effect of −21·7 gal.

49·6 % TfA/D cows with genetic effect of 0 gal.

20·5 % TfD/D cows with genetic effect of +21·7 gal.

The variance in milk yield due to the transferrin locus is therefore

$$((29{\cdot}9 + 20{\cdot}5) \times 21{\cdot}7^2)/100 = 237{\cdot}3 \text{ gal.}$$

Accordingly an estimate of the proportion of the genetic variance in milk yield in this population ascribable to the transferrin locus is

$$(237{\cdot}3 \times 100)/2288 = 10{\cdot}4\,\%.$$

In the Illawarra Shorthorn population of 433 cows the mean milk yield was 488·2 gallons, with a phenotypic standard deviation of ± 117·86 gallons. Assuming a heritability of 0·3 the genetic variance due to milk yield is 4167 gal.^{2}. The gene frequencies in the population were $TfA = 0{\cdot}316$, $TfD = 0{\cdot}602$, and $TfE = 0{\cdot}082$. The variance in milk yield due to replacing TfA by TfD at the transferrin locus was calculated to be 248·8 gallons. Accordingly the proportion of the genetic variance ascribable to the transferrin locus in the Illawarra Shorthorn population is about 6·0 %.

It is interesting that homozygosity for TfD, if attainable in practice, would raise yield by about 21 to 23 gallons in these populations. This implies that the manipulation of a single gene contributing 6 or 10 % to genetic variance could raise yield by about 5 % in two generations, say 10 years, in these Queensland herds by rather simple selection. This compares with an expectation of about 1 % per annum by ordinary selection (Rendel & Robertson, 1950) and would of course be additional to the improvement achieved by such selection.

It is pertinent to inquire if selection for TfD would cause adverse effects due to the elimination of heterozygotes from the population. Heterozygotes are at an advantage *in utero* (Ashton & Fallon, 1962), but this advantage is outweighed by the higher breeding efficiency obtained between homozygous partners, presumably due to superior fertilization efficiency of such types. On balance, therefore, it would seem that selection for TfD would improve milk yield and fertility at the same time.

The nature of the effect. It is not yet known why the iron-binding serum β-globulins (transferrins) influence production characteristics. There is circumstantial evidence that the effect is not solely due to increased lactation length.

Recent evidence points to immunological autonomy between certain serum proteins and milk proteins (Larson, 1958; Hanson, 1959), and it is interesting that Gahne, Rendel & Venge (1960) have found apparently unchanged transferrins in samples of milk. At the moment it is not apparent how the capacity of the transferrins to bind iron is concerned with their known genetic effects.

SUMMARY

1. The transferrin types of 225 Jersey cows in thirteen herds, and 433 Australian Illawarra Shorthorn cows in twenty-four herds in Queensland were determined. The effect of transferrin type on lactation length, milk yield, butterfat yield and butterfat percentage was assessed by a least-squares multiple linear regression analysis.

2. The results for the two breeds did not differ significantly. Combining the data from both breeds, it was found that on average D/D cows had

lactations 13·9 days longer than A/A cows ($P < 0·01$), and yielded 449·7 lb. more milk ($P < 0·01$). The results for A/D cows were intermediate.

3. The proportion of the genetic variance in milk yield due to the transferrin locus was 10·4 % in the Jerseys and 6·0 % in the Australian Illawarra Shorthorns. This implies that the manipulation of a single gene contributing 6–10 % to genetic variance could raise milk yield by about 5 % in two generations in these Queensland herds by rather simple selection.

4. The nature of the effect is not known, but it does not seem to be due solely to increased length of lactation.

We wish to thank Messrs J. M. Crofts and J. T. Piggott of the Queensland Department of Agriculture and Stock for supplying blood samples, Dr P. J. Claringbold for organizing the computer analysis and for statistical advice, and Dr A. Robertson and Mr H. G. Turner for useful criticism of the manuscript.

REFERENCES

ASHTON, G. C. (1958). *Nature, Lond.*, **182**, 370.
ASHTON, G. C. (1960). *J. Agric. Sci.* **54**, 321.
ASHTON, G. C. (1961). *J. Reprod. Fert.* **2**, 117.
ASHTON, G. C. & FALLON, G. R. (1962). *J. Reprod. Fert.* **3**, 93.
GAHNE, B., RENDEL, J. & VENGE, O. (1960). *Nature, Lond.*, **186**, 907.
GIBLETT, E. R., HICKMAN, C. G. & SMITHIES, O. (1959). *Nature, Lond.*, **183**, 1589.
HANSON, L. A. (1959). *Experientia*, **15**, 471.
JOHANSSON, I. (1950). *Anim. Breed. Abstr.* **18**, 1.
LARSON, B. L. (1958). *J. Dairy Sci.* **41**, 1033.
RENDEL, J. M. & ROBERTSON, A. (1950). *J. Genet.* **50**, 1.
ROBERTSON, A. & RENDEL, J. M. (1954). *J. Agric. Sci.* **44**, 184.
SMITHIES, O. (1955). *Biochem. J.* **61**, 629.
SMITHIES, O. & HICKMAN, C. G. (1958). *Genetics*, **43**, 374.

Genet. Res., Camb. (1965), 6, *pp.* 209–215
Printed in Great Britain

Transferrin and post-albumin polymorphism in East African cattle

By G. C. ASHTON*

Cattle Research Laboratory, C.S.I.R.O., Rockhampton, Queensland, Australia

and G. H. LAMPKIN

East African Veterinary Research Organization, Muguga, Kikuyu, Kenya

(*Received* 23 *June* 1964)

1. INTRODUCTION

Gene frequencies for the three transferrin alleles in European breeds of beef and dairy cattle are reasonably well established (Ashton, 1958; Schmid, 1962). Comparable frequencies for Zebu breeds have been reported so far only by Ashton (1959) and then only from small numbers of Zebu cattle imported into Australia, and their progeny from crosses with European cattle. This paper reports data for some East African breeds. Post-albumin phenotypes (Ashton, 1963) were also determined on the samples available, and gene frequencies for the polymorphism calculated for the breeds examined.

The cattle chosen for sampling were from herds which were considered likely to represent the breed as a whole. However, the gene frequencies presented here are herd gene frequencies and may be only an approximation to the true breed gene frequencies.

2. MATERIALS AND METHODS

Breeds sampled represented the main cattle types found in East Africa as listed by Mason (1951). Sanga cattle were represented by the Ankole breed, East African Shorthorned Zebus by the Tanganyika Shorthorned Zebu, Boran and Teso (Bukedi) breeds and Sanga × Shorthorned Zebu by the Nganda breed. In addition a herd of Sahiwal (Indian Zebu) cattle resident in Kenya was examined.

The 267 serum samples from Ankole cattle were collected from two farms belonging to the Uganda Department of Veterinary Services and Animal Industry at Mbarara and Ruhengere. Both these herds had been derived from a common source and throughout this paper the data resulting from the Ankole samples have been treated as if they were derived from one herd. The Teso and Nganda cattle also belonged to the Uganda Department. The seventy-two samples that were obtained from the former came from a foundation herd maintained at the Livestock Husbandry Experimental Unit, Nakyesasa, whilst the 138 samples from Nganda

* Present address: Department of Genetics, University of Hawaii, Honolulu 14, Hawaii, U.S.A.

Table 1. *Number of transferrin phenotypes observed in six populations of East African cattle, and number expected calculated from the gene frequencies in Table 2*

Breed	Obs. or exp.	Phenotype															
		AA	AB	AD	AF	AE	BB	BD	BF	BE	DD	DF	DE	FF	FE	EE	Total
Sahiwal	Obs.	1	4	8	12	5	2	7	17	12	12	46	27	29	38	8	228
	Exp.	1·05	2·99	7·62	11·63	6·66	2·12	10·81	16·50	9·46	13·75	42·00	24·07	32·06	36·75	10·53	228·0
Nganda	Obs.	20	11	10	29	26	0	4	4	6	3	2	10	0	11	2	138
	Exp.	24·38	10·50	13·44	19·34	23·95	1·13	2·90	4·17	5·16	1·85	5·33	6·61	3·83	9·50	5·88	138·0
Boran	Obs.	1	3	9	13	15	1	3	18	11	11	37	31	36	52	22	263
	Exp.	1·67	2·95	8·14	15·32	12·21	1·30	7·17	13·50	10·76	9·89	37·23	29·67	35·04	55·85	22·26	263·0
Tanganyika Shorthorned Zebu	Obs.	4	0	12	19	12	1	0	5	1	3	9	7	17	22	2	114
	Exp.	5·70	1·79	7·60	19·91	10·29	0·14	1·19	3·12	1·61	2·53	13·27	6·86	17·37	17·95	6·64	114·0
Teso	Obs.	1	2	5	11	6	0	0	0	3	0	6	6	7	16	9	72
	Exp.	2·35	0·90	3·07	8·49	8·85	0·09	0·39	1·63	1·70	1·00	5·55	5·78	7·67	15·99	8·34	72·0
Ankole	Obs.	14	12	7	30	49	3	3	10	16	1	13	22	8	45	34	267
	Exp.	14·87	11·09	11·09	26·91	47·20	2·07	4·14	10·03	17·60	2·07	10·03	17·60	12·17	42·70	37·45	267·0

cattle came from the Animal Health Research Centre at Entebbe. The Boran herd from which 265 samples were obtained was the one kept at Muguga by the East African Veterinary Research Organization, the breeding of which has been described by Lampkin & Lampkin (1960). The 114 samples obtained from the Tanganyika Shorthorned Zebus came from the Tanganyika Government Livestock Research Centre at Mpwapwa, whilst the 228 Sahiwal samples were obtained from the National Sahiwal Stud maintained by the Kenya Department of Veterinary Services at Naivasha, a station situated about 50 miles west of Nairobi.

Starch gel electrophoresis

The serum samples were examined by starch gel electrophoresis in a discontinuous tris-citric, boric acid-lithium hydroxide system. Details of this procedure have been published (Ashton & Lampkin, 1965).

3. RESULTS

Transferrins

All fifteen phenotypes described by Ashton (1959) were found in the cattle sera examined, representing the homozygotes and heterozygotes of the alleles Tf^A, Tf^B, Tf^D, Tf^E and Tf^F. In addition a further phenotype Tf GF (Ashton & Lampkin, 1964) was found in three Borans. These animals have not been included in the analysis of the transferrin results because of the infrequency of this phenotype.

Table 1 shows the numbers of each transferrin phenotype observed in each of the breeds examined. Table 2 shows the gene frequencies derived from these

Table 2. *Transferrin gene frequencies and standard errors for six populations of East African cattle, calculated from the observed phenotype distribution shown in Table* 1

Breed	Tf^A	Tf^B	Tf^D	Tf^F	Tf^E
Sahiwal	0·068	0·096	0·246	0·375	0·215
	±0·012	±0·014	±0·020	±0·023	±0·019
Nganda	0·420	0·091	0·116	0·167	0·206
	±0·030	±0·017	±0·019	±0·022	±0·024
Boran	0·080	0·070	0·194	0·365	0·291
	±0·012	±0·011	±0·017	±0·021	±0·020
Tanganyika	0·224	0·035	0·149	0·390	0·202
Shorthorned Zebu	±0·030	±0·011	±0·023	±0·032	±0·023
Teso	0·181	0·035	0·118	0·326	0·340
	±0·032	±0·015	±0·027	±0·039	±0·040
Ankole	0·236	0·088	0·088	0·214	0·374
	±0·018	±0·012	±0·012	±0·018	±0·021

data, and their standard errors. Table 1 also shows the numbers of each phenotype expected, assuming genetic equilibrium, calculated from the derived gene frequencies.

Post-albumins

The three post-albumin phenotypes PaF, PaFS, and PaS found in European cattle (Ashton, 1963) were represented in each breed examined except Nganda. In this breed only PaF and PaFS types were found.

Table 3 shows the numbers of each post-albumin phenotype found in each of the breeds examined. The three Borans with transferrin type TfGF were included in Table 3, but one other Boran sample could not be typed on initial electrophoresis

Table 3. *Number of post-albumin phenotypes observed in six populations of East African cattle, and number expected calculated from the gene frequencies in Table* 4

Breed	Obs. or exp.	Phenotype F	FS	S	Totals
Sahiwal	Obs.	49	106	73	228
	Exp.	45·64	112·74	69·62	228·0
Nganda	Obs.	104	34	0	138
	Exp.	106·09	29·82	2·09	138·0
Boran	Obs.	121	123	21	265
	Exp.	125·69	113·63	25·68	265·0
Tanganyika Shorthorned Zebu	Obs.	82	29	3	114
	Exp.	81·69	29·62	2·69	114·0
Teso	Obs.	39	29	4	72
	Exp.	39·76	27·49	4·75	72·0
Ankole	Obs.	120	121	26	268
	Exp.	122·01	116·96	28·03	268·0

and the sample was lost before it could be repeated. Gene frequencies and their standard errors derived from Table 3 are shown in Table 4. Table 3 also shows the numbers of each phenotype expected, assuming genetic equilibrium, calculated from the gene frequencies.

Table 4. *Post-albumin gene frequencies and standard errors for six populations of East African cattle, calculated from the observed phenotype distribution shown in Table* 3

Breed	Pa^F	Pa^S
Sahiwal	0·447 ± 0·023	0·553 ± 0·023
Nganda	0·877 ± 0·020	0·123 ± 0·020
Boran	0·689 ± 0·020	0·311 ± 0·020
Tanganyika Shorthorned Zebu	0·847 ± 0·024	0·153 ± 0·024
Teso	0·743 ± 0·037	0·257 ± 0·037
Ankole	0·676 ± 0·020	0·324 ± 0·020

4. DISCUSSION

Table 2 shows that each of the five transferrin alleles previously found in Sindhi and Sahiwal dairy cattle (Ashton, 1959) were represented in each of the breeds examined. In addition a further allele, Tf^G, was found in three of the Borans (Ashton & Lampkin, 1965). These East African cattle differ markedly from European cattle in transferrin constitution, having three alleles Tf^B, Tf^F, and Tf^G not found in European breeds.

only a few sires could have accounted for this. In all the breeds, however, Tf^B was very infrequent (less than 10%). The reasons for the predominance of Tf^E and Tf^F types are not known. In nearly all European breeds examined Tf^E is the least frequent allele (see, for example, Schmid, 1962), and in some breeds (Jersey, Guernsey) Tf^E may be absent in some locations.

The frequencies for the two post-albumin alleles (Table 4) were similar for all breeds except Sahiwal. Pa^F is two to three times more frequent than Pa^S, except in Sahiwals where Pa^S is slightly more frequent than Pa^F. These frequencies differ markedly from European breeds where Pa^S is the predominant allele, and the frequency of Pa^F is low or zero (Ashton, unpublished observations).

Both polymorphic systems examined in these East African cattle show marked differences from European breeds. It is speculative whether Tf^B, Tf^F, Tf^E, and Pa^F are more frequent in Zebu cattle because they have special advantage for these animals in their native environment, or whether the gene frequency difference is merely a reflexion of the genotype of the ancestors of the two types of cattle.

Maintenance of a stable polymorphism is commonly brought about by superior fitness of the heterozygotes, resulting in a relative excess of heterozygotes in the population. Evidence suggesting superior fitness of the common heterozygote in European dairy cattle comes from the time distribution of returns-to-service following insemination. Ashton & Fallon (1962) found that matings between TfAA and TfDD parents, producing only TfAD offspring, gave 21·8% of the service returns 25 days or later following insemination. The mean figure for all other matings was about 31%. A similar effect has been observed by the New Zealand Dairy Production and Marketing Board (1963). TfAA × TfDD matings gave 16% of service returns in the period 25–49 days after insemination, while the mean figure for all other matings was about 27%. Assuming that long returns (i.e. post 25 days) bear a relationship to embryonic loss this implies that heterozygotes are more viable *in utero*. A relative excess of heterozygotes would be expected in a dairy cattle population as a result.

The data in Table 5 show that 4·2% more transferrin heterozygotes were found than expected by calculation. An excess of heterozygotes is normally obtained by this method of calculation and amounts to 1/8 S, where S is the number of sires in use in the herds sampled (Robertson, 1965). The number of effective sires in these East African herds is not known, but will have been the same for both transferrin and post-albumin systems. The excess of heterozygotes at the post-albumin locus was 2·7%. If it is assumed that this is due solely to the limited use of sires and to the method of calculation then this is the approximate extent of the excess expected at the transferrin locus. The difference of 1·5% between the two heterozygote excesses is not significant, but suggests that a true excess of heterozygotes may have been present in these East African cattle. Further surveys in which concurrent analyses of transferrin and other serum polymorphisms are made may throw light on the difficult problem of determining whether or not a transferrin heterozygote excess exists in cattle populations. A survey in 293 Droughtmaster cattle in Queensland (Ashton, Francis & Ritson, unpublished data) gave a

transferrin heterozygote excess of 7·03% and a post-albumin heterozygote excess of 2·54%.

The extent of the transferrin heterozygote excess may be quite small, and yet sufficient to maintain a stable polymorphism. It is known that heterozygote fitness superiority of the order of 1% is sufficient (Blumberg, 1961). In a two-allele system

Table 5. *Homozygotes and heterozygotes observed and expected for transferrin and post-albumin polymorphism in six populations of East African cattle*

	Transferrins				Post-albumins			
	Homozygotes		Heterozygotes		Homozygotes		Heterozygotes	
Breed	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Sahiwal	52	59·51	176	168·49	122	115·26	106	112·74
Nganda	25	37·07	113	100·93	104	108·18	34	29·82
Boran	71	70·16	192	192·84	142	151·37	123	113·63
Tanganyika Shorthorned Zebu	27	30·38	87	83·62	85	84·38	29	29·62
Teso	17	19·45	55	52·55	43	44·51	29	27·49
Ankole	60	68·63	207	198·37	146	150·04	121	116·96
Totals	252	285·20	830	796·80	642	653·74	442	430·26

a loss of 5% of homozygotes from all matings in which equal numbers of homozygotes and heterozygotes would be expected will lead to a true heterozygote excess of 1·9% in the population by the usual method of calculation.

SUMMARY

The serum transferrin and post-albumin phenotype distributions of 228 Sahiwal, 138 Nganda, 265 Boran, 114 Tanganyika Shorthorned Zebu, 72 Teso and 267 Ankole cattle from East Africa were determined.

Five transferrin alleles, Tf^A, Tf^B, Tf^D, Tf^E, and Tf^F were present in all the breeds examined, and a sixth allele, Tf^G, was present in three of the Borans. Tf^E and Tf^F were the most frequent alleles, except in the Nganda cattle where Tf^A was the most frequent. Tf^B had a frequency of less than 0·1 in each breed. Two post-albumin alleles, Pa^F and Pa^S, were present in each breed. In each breed except Sahiwal Pa^F was two to three times more frequent than Pa^S. In the Sahiwal Pa^F and Pa^S had about the same frequency. It was concluded that both transferrin and post-albumin gene frequencies in East African cattle differ significantly from the corresponding frequencies in European cattle.

There was no evidence of an excess of heterozygotes in the post-albumin system other than that expected from the use of relatively small numbers of bulls in these herds. However, allowing for the same factor in the transferrin system, there appeared to be an excess of transferrin heterozygotes in the cattle populations sampled although the extent of this excess could not be calculated accurately.

Thanks are due to the Director of the East African Veterinary Research Organization for the facilities made available to Dr G. C. Ashton, whilst working as a guest with the Organization and to the staffs of the Kenya, Uganda and Tanganyika Veterinary Departments who assisted in collecting and supplying samples.

REFERENCES

ASHTON, G. C. (1958). Genetics of β-globulin polymorphism in British cattle. *Nature, Lond.* **182**, 370–372.

ASHTON, G. C. (1959). β-Globulin alleles in some Zebu cattle. *Nature, Lond.* **184**, 1135–1136.

ASHTON, G. C. (1963). Polymorphism in the serum post-albumins of cattle. *Nature, Lond.* **198**, 1117–1118.

ASHTON, G. C. & FALLON, G. R. (1962). β-Globulin type, fertility, and embryonic mortality in cattle. *J. Reprod. Fert.* **3**, 93–104.

ASHTON, G. C. & LAMPKIN, G. H. (1965). Serum albumin and transferrin polymorphism in East African cattle. *Nature, Lond.* **205**, 209–210.

BLUMBERG, B. S. (ed.) (1961). *Proceedings of the Conference on Genetic Polymorphisms and Geographic Variations in Disease.* New York: Grune and Stratton.

LAMPKIN, G. H. & LAMPKIN, K. (1960). Studies on the production of beef from Zebu cattle in East Africa. I. A description of the Muguga herd. *J. agric. Sci., Camb.* **55**, 229–231.

MASON, I. L. (1951). A world dictionary of breed types and varieties of livestock. *C'wealth Agric. Bureaux, Farnham Royal, Bucks.*

NEW ZEALAND DAIRY PRODUCTION AND MARKETING BOARD (1963). Farm Production Report No. 39, 1962–63 season, p. 54.

ROBERTSON, A. (1965). The interpretation of segregation ratios in domestic animal populations. *Anim. Prod.* (in press).

SCHMID, D. O. (1962). Die genetische Bedeutung erblicher Serumeiweissmerkmale bei Tieren. *Tierärztl. Umsch.* **17**, 302–314.

Reprint from GENETICS, Vol. 52, No. 5, November, 1965. *Printed in U.S.A.*

CATTLE SERUM TRANSFERRINS: A BALANCED POLYMORPHISM?

G. C. ASHTON

Department of Genetics, University of Hawaii, Honolulu

Received June 14, 1965

FISHER (1930) defined the most likely condition for a stable polymorphism involving two alleles as one in which the heterozygote showed superior fitness relative to both homozygotes. The conditions for stability of a polymorphism in which more than two alleles occur have been examined by several authors (for review see SMITH 1961). There is general agreement that overdominance is a prerequisite for maintaining stability. A number of polymorphisms are known in invertebrates in which heterozygote advantage has been demonstrated experimentally (FORD 1964; SHEPPARD 1958). In vertebrates heterozygote advantage has been established with certainty as a balancing mechanism only in the case of the sickle cell hemoglobin heterozygote (ALLISON 1956), although there is mounting evidence (MORTON and CHUNG 1959) that the human MN blood group polymorphism may also be maintained by heterozygote advantage.

So far heterozygote advantage has not been demonstrated for any of the serum protein polymorphisms now known in vertebrates. ASHTON (1958) reported that differential fertility might be occurring between matings of various cattle serum transferrin genotypes. Matings between parents from which equal numbers of offspring like and unlike the mother (with respect to transferrin genotype) were expected showed a significant excess of like-mother progeny. Other workers were not able to confirm this result (BRUMMERSTEDT-HANSSEN, MOUSTGAARD and MØLLER 1963), but the results of the first segregation analysis prompted a direct investigation of the breeding efficiency of matings between parents of known transferrin genotype (ASHTON 1961). Pooled data for 2544 inseminations are shown in Table 1. The most successful matings were from homozygous parents of unlike genotype (61.6%), followed by matings between homozygous parents of like genotype (55.6%). Matings involving heterozygotes were less efficient, homozygotes × heterozygotes giving a mean breeding efficiency of 49.2%, and matings between two heterozygotes giving 45.6%. Because Tf^E was absent from the Jersey cattle examined, and at low frequency in the Australian Illawarra Shorthorns, the data in Table 1 refer only to matings involving the alleles Tf^A and Tf^D. Although these results seemed to show heterozygotes at a disadvantage, subsequent analysis of the returns-to-service data (ASHTON and FALLON 1962) indicated that heterozygotes were more viable *in utero* and therefore at an advantage (see below).

Genetics Paper No. 5.43. Contribution from Pacific Biomedical Research Center

This investigation was supported in part by Public Health Service Research Grant 1 RO1 HD 01831-01, from the National Institute of Child Health and Human Development.

TABLE 1

Breeding efficiencies (percentage of inseminations resulting in a live or stillborn calf) for matings between various transferrin genotypes

Cow transferrin genotype	Bull transferrin genotype		
	A/A	*A/D*	*D/D*
A/A	55.9	45.3	59.5
A/D	51.0	45.6	46.9
D/D	61.9	50.3	55.3

Data from ASHTON and FALLON, (1962).

Consideration of data from several sources suggests that four phenomena affecting reproductive performance are associated with transferrin polymorphism in cattle. These are (1) superiority of heterozygotes *in utero*, (2) mother-fetus incompatibility, (3) differential fertility of heterozygous and homozygous bulls, and (4) differential fertility of heterozygous and homozygous cows. Interaction between these factors produces the observed distribution of breeding efficiencies. Notwithstanding the relatively poor breeding efficiency of matings involving heterozygous parents it can be shown that the advantage of the heterozygote *in utero* may be sufficient to produce a balanced polymorphism.

Heterozygote superiority: Because Tf^E is relatively infrequent, or even absent, in European breeds of cattle the data available refer mainly to Tf^A and Tf^D, and only these alleles are considered in the main treatment in this paper. It is now known that "Tf^D" is really two alleles Tf^{D1} and Tf^{D2} (KRISTJANSSON 1962; ASHTON 1965). Nearly all the published segregation and fertility data treat these two alleles as a single Tf^D, and it is necessary to remember that "TfAD" can be either TfAD1 or TfAD2, and "TfDD" can be TfD1D1, TfD1D2, or TfD2D2.

Evidence for superiority of the transferrin heterozygote TfAD comes from three sources:

(a) *Returns-to-service data.* Table 2 shows the distribution of unsuccessful inseminations as evidenced by the reappearance of oestrus ("return-to-service") for

TABLE 2

Returns-to-service in artificially inseminated cows from two sources

Source	Mating	Total inseminations	Returns					
			0–24 days			25 days or more		
			No.	Percent total	χ^2	No.	Percent total	χ^2
ASHTON and FALLON (1962)	Like homozygotes	426	131	30.8	...	58	13.6	...
	Unlike homozygotes	273	82	30.0	0.04	23	8.4	4.37*
New Zealand D. P. M. B. (1963)	Like homozygotes	101	28	27.7	...	10	9.9	...
	Unlike homozygotes	119	29	24.4	0.32	5	4.2	2.79

* $P<0.05$.

the two time periods 0–24 days, and 25 days or longer, after insemination. Most of the inseminations which do not result in conception tend to occur in the first period. Returns in the second period ("long returns") are partly or mainly composed of inseminations which caused conception but from which the products of conception did not remain viable. There is of course considerable overlap between the two categories (ASHTON and FALLON 1962). Two sets of data only have been published which report returns-to-service. These are shown in Table 2. Each set shows that long returns are least frequent from matings between unlike homozygotes i.e., TfAA male × TfDD female and TfDD male × TfAA female. Analysis by χ^2 (SNEDECOR 1956) shows that the effect is significant in the Australian data ($P < 0.05$) but not in the New Zealand data. However the two sets of data are not significantly heterogeneous with respect to the distribution of long returns ($\chi^2 = 0.57$, 2 d.f., $P > 0.7$) and the difference in the pooled data is significant ($\chi^2 = 5.88$, 1 d.f., $P < 0.02$).

These results imply that heterozygotes, produced from matings between unlike homozygous parents, are more likely to survive *in utero* than homozygotes, produced from matings between like-type homozygous parents.

It would be anticipated that matings producing homozygotes and heterozygotes in equal numbers would show an intermediate frequency of long returns. However, as shown later some of these matings are subject to maternal-fetal incompatibility and it is not possible to separate the loss due to the two causes by an examination of long returns data.

(b) *Matings between like heterozygotes.* Direct evidence of heterozygote superiority comes from analysis of segregation data from matings expected to produce homozygotes and heterozygotes in equal numbers i.e., A/D male × A/D female, A/A male × A/D female, and D/D male × D/D female. In these matings the mother will possess both alleles present in the fetus, and incompatibility will not be a complicating factor. Several workers have now reported segregation data, and their results are shown in Table 3 for the three relevant matings. The data were analyzed by the method given in SNEDECOR (1956). Overall, 717 heterozygotes and 637 homozygotes were produced, which differs significantly from equality ($\chi^2 = 4.73$, 1 d.f., $P < 0.05$). There was no significant heterogeneity between matings with regard to the distribution of homozygotes and heterozygotes ($\chi^2 = 0.65$, 2 d.f., $P > 0.7$). Further data are necessary before the extent of the effect can be established with certainty, but the weighted data in Table 3 imply that only about 89% of the homozygotes expected were produced by these matings.

(c) *Heterozygote excess in cattle populations.* A third type of evidence which would suggest heterozygote advantage would be observation of more heterozygotes in random samples of cattle populations than expected from the gene frequencies. ASHTON and FALLON (1962) summarized data showing a significant excess ($P < 0.02$) of about 6% of heterozygotes in several cattle populations. It is not easy to assess data of this sort, because cattle are not usually randomly bred, and moreover not every survey shows such an excess. An artefactual excess of heterozygotes will be obtained by the usual method of computing expected dis-

TABLE 3

Distribution of homozygotes and heterozygotes from matings in which maternal fetal incompatibility is not possible

Mating ♂ ♀	Source*	Progeny		
		Homozygotes	Heterozygotes	Total
A/D × *A/D*	A	198	231	429
	B	62	72	134
	C	23	26	49
	D	0	2	2
	E	26	31	57
	F	36	35	71
	Total	366	420	786
A/A × *A/D*	A	105	98	203
	B	26	17	43
	C	6	6	12
	E	25	42	67
	F	0	6	6
	Total	162	169	331
D/D × *A/D*	A	61	66	127
	B	15	16	31
	C	7	6	13
	D	3	3	6
	E	13	22	35
	F	10	15	25
	Total	109	128	237
TOTALS		637	717	1354

* *Source key:* A, BRUMMERSTEDT-HANSSEN *et al.* (1963). Progeny test data from 2079 matings (their Table 4). B, BRUMMERSTEDT-HANSSEN *et al.* (1963). Data relating to bull calves (their Table 6). C, GAHNE (1960). D, GALL and BERG (1964). E, ASHTON (1958b). F, ASHTON and FALLON (1962).

tributions of genotypes, owing to the restricted use of sires (ROBERTSON 1965) in dairy and beef cattle herds. This difficulty could be resolved by working within sire groups, but in most cases the relevant information necessary to extract sire groups is not presented.

An alternative approach is to compare within the same population the distribution of transferrin homozygotes and heterozygotes with another genetic system which does not show significant deviation from a Hardy-Weinberg distribution. In this way the extent of heterozygote excess due to limited use of sires should be expressed in both systems. If a consistent extra excess of heterozygotes is found for the transferrin system and not for other systems this would imply a true excess of transferrin heterozygotes in cattle populations. Relevant data are shown in Table 4 contrasting the transferrin and post-albumin (ASHTON 1963) genotype distributions for several populations of cattle of different breeds. There was a significant excess of transferrin heterozygotes ($P < 0.01$) but not of post-albumin heterozygotes ($P > 0.5$). There were on average 5.5% more transferrin heterozygotes than expected, and 2.5% more post-albumin heterozygotes. The

TABLE 4

Expected and observed distributions of transferrin and post-albumin heterozygotes from surveys of several cattle populations

Source*	Population	Transferrin heterozygotes		Post-albumin heterozygotes	
		Observed	Expected	Observed	Expected
G	Sahiwal	176	168	106	113
	Nganda	113	101	34	30
	Boran	192	193	123	114
	Tanganyika zebu	87	84	29	30
	Teso	55	53	29	27
	Ankole	207	198	121	117
H	Steers	43	36	30	27
	Breeders (Classified)	79	72	46	46
	Breeders (Unclassified)	119	113	74	72
	Brisbane	100	92	61	61
		1171	1110	653	637

* *Source Note:* G from ASHTON and LAMPKIN (1965); H from ASHTON, FRANCIS and RITSON, unpublished data.

value of 5.5% for transferrin heterozygote excess for these herds is close to the 6.2% excess found in other surveys (ASHTON and FALLON 1962).

Maternal-fetal incompatibility: ASHTON (1958a) described an excess of like-mother offspring from matings involving the alleles Tf^A and Tf^D suggesting mother-fetus incompatibility. Other workers failed to confirm this effect, but it is nevertheless clear from their results that incompatibility exists. Segregation from matings which have an equal chance of producing progeny compatible or incompatible with the mother are shown in Table 5 for all the available data. Overall, these matings produced 478 compatible and 409 incompatible progeny, equal numbers of each being expected ($\chi^2 = 5.36$, $P < 0.05$). The distribution of compatible and incompatible progeny was not significantly heterogenous ($\chi^2 = 0.74$, 1 d.f., $P > 0.3$) between mating groups.

The ratio between incompatible and compatible progeny is 0.86 which suggests that only 86% of incompatible progeny survive. However, it is impossible to derive a true estimate for incompatibility because all matings of this type produce heterozygotes which may be at an advantage *in utero*, and this will confound the incompatibility effect.

Heterozygote fertility: Apart from the two effects of transferrin type on fitness considered above, there is evidence that the fertility of heterozygous bulls is less than that of homozygous bulls, irrespective of cow genotype. Also there is less substantial evidence of a similar effect with respect to homozygous and heterozygous cows.

Data relevant to bull fertility are shown in Table 6. These relate to TfAD bulls compared with pooled data for TfAA and TfDD bulls, bulls with Tf^E being relatively infrequent. In assessing the relative fertility of bulls of different genotypes

TABLE 5

Distribution of compatible and incompatible progeny from matings where equal numbers of both types are expected

Mating ♂ ♀	Source*	Progeny		
		Compatible	Incompatible	Total
A/D × *D/D*	A	99 *D/D*	78 *A/D*	177
	B	30	18	48
	C	13	12	25
	D	5	4	9
	E	41	29	70
	F	22	27	49
	Total	210	168	378
A/D × *A/A*	A	159 *A/A*	134 *A/D*	293
	B	55	59	104
	C	12	14	26
	D	7	4	11
	E	20	12	32
	F	15	28	43
	Total	268	241	509
TOTAL		478	409	887

* *Source key:* See Table 3.

TABLE 6

Relative fertility of homozygous (mainly TfAA and TfDD) and heterozygous (mainly TfAD) bulls

Source	Fertility parameter	Bull fertility		Ratio (a)/(b)
		Homozygotes (a)	Heterozygotes (b)	
Ashton (1961)				
Jersey bulls	Live and stillbirths	54.8	43.6	1.257
A.I.S. bulls	Live and stillbirths	54.0	47.1	1.146
Ashton and Hewetson (unpublished data)				
Jersey × zebu bulls	Live and stillbirths	62.2	57.5	1.082
Datta (1963)				
Blaine herd	Live births	57.5	53.8	1.069
D.C.R.C. herd	Live births	45.0	33.7	1.335
Hickman and Dunn (1961)				
Holstein bulls	Non-returns (period unstated)	74.3	74.4	0.999
Ayreshire bulls	Non-returns (period unstated)	76.9	73.8	1.042
Jersey bulls	Non-returns (period unstated)	75.5	75.5	1.000
Guernsey bulls	Non-returns (period unstated)	69.7	64.8	1.076
Angus bulls	Non-returns (period unstated)	78.2	79.0	0.989
New Zealand D.P.M.B. (1963)	49-day non-returns	65.0	64.5	1.008

the major problem is the small number of bulls usually available for comparison. Large numbers of inseminations for a single bull will increase the precision of the estimate of that bull's fertility, but will not contribute to the estimate of the mean fertility for bulls of a given genotype, which is the main interest. Table 6 shows that in the data so far published, TfAD bulls are mostly less fertile than TfAA and TfDD bulls. The extent of the effect varies, and it is not possible to get an accurate estimate of the difference in fertility of the two types of bulls from Table 6. It is necessary to weight the ratios for homozygote:heterozygote fertility by the number of bulls of each type used in deriving the ratios, and this information is not available for all these data.

The poorer fertility of heterozygous bulls is substantiated in another way. Fertility is an important factor in bulls standing at Artificial Breeding Centers, and it might be anticipated that an excess of homozygous bulls would be found at such centers as a result of bull selection. The distribution of 766 bulls of seven breeds, standing at more than 20 centers in the United Kingdom in the period 1956–57, is shown in Table 7. The only other data relating to bulls at Artificial Breeding Centers were published by GAHNE (1961), for 404 SRB bulls in Sweden, and these show the same effect. Analysis of the data in Table 7 by χ^2 (SNEDECOR 1956) showed a significant difference between the expected and observed distribution of homozygous and heterozygous bulls ($\chi^2 = 8.61$, 1 d.f., $P < 0.01$) and gave no evidence of heterogeneity between breeds ($\chi^2 = 8.60$, 7 d.f., $P > 0.2$). There were 18.9% more homozygous than heterozygous bulls based on expected distributions. This contrasts with the excess of heterozygotes found in cow populations (Table 4) in which selection for milk yield rather than fertility is usual.

Data showing the relative fertility of homozygous and heterozygous cows are given in Table 8 and relate largely to TfAA, TfAD and TfDD cows. They support the conclusion that TfAD cows are less fertile than the homozygous cows, but the effect is not significant. Analysis by WOOLF's (1955) method gave χ^2 for

TABLE 7

Observed and expected distributions of homozygous (TfAA, TfDD and TfEE) and heterozygous (TfAD, TfAE and TfDE) bulls standing at Artificial Breeding Centers

		Bull genotype			
		Homozygotes		Heterozygotes	
Source	Breed	Observed	Expected	Observed	Expected
ASHTON, 1958b (United Kingdom)	Jersey	29	28	20	21
	Guernsey	32	31	30	31
	Angus	29	26	23	26
	Ayreshire	59	61	65	63
	Hereford	52	39	25	38
	Shorthorn	82	72	59	69
	Friesian	135	119	126	142
GAHNE (1961) (Sweden)	S.R.B.	168	160	236	244
Totals		586	536	584	634

TABLE 8

Relative fertility of heterozygous (mainly TfAD) and homozygous (mainly TfAA and TfDD) cows

Source	Fertility parameter	Cow fertility			
		Homozygotes		Heterozygotes	
		No. of inseminations	Percent successful	No. of inseminations	Percent successful
ASHTON (1961)					
Jersey cows	Live and stillbirths	746	52.6	781	49.6
A.I.S. cows	Live and stillbirths	476	56.3	690	45.7
New Zealand D.P.M.B. (1963)	49-day non-returns	443	66.1	393	63.1
DATTA (1963)					
Blaine cows	Live births	225	51.6	206	60.2
D.C.R.C. cows	Live births	112	40.2	143	41.3
ASHTON and HEWETSON (unpublished data)	Live and stillbirths	477	60.0	387	59.4

deviation of incidence ratio from unity = 1.58, which is not significant, and χ^2 for heterogeneity = 4.52, 5 d.f., $P > 0.5$. The analysis showed that homozygous cows were about 6.2% more fertile than heterozygous cows on average.

The proposed model: A model which fits the order of breeding efficiencies obtained by ASHTON and FALLON (1962) is shown in Table 9. This model utilizes the four demonstrated effects of transferrin type on fertility and is developed for the two alleles Tf^A and "Tf^D", little being known about Tf^E, or the two individual *D* alleles.

(1) *Heterozygote superiority.* Matings between unlike homozygotes which

TABLE 9

Model proposed to explain the observed distribution of breeding efficiencies from the pooled data of Ashton and Fallon (1962)

Mating ♂ ♀	Observed		Model				
			Weighting factors				
	Observed breeding efficiency (percent)	Ratio obs.*	Heterozygote superiority	Incompatibility	Bull fertility	Cow fertility	Ratio expected†
AA × *AA*	55.9	0.90	0.85	1.00	1.0	1.0	0.94
AA × *AD*	51.0	0.82	0.92	1.00	1.0	0.9	0.91
AA × *DD*	61.9	1.00	1.00	0.90	1.0	1.0	1.00
AD × *AA*	45.3	0.73	0.92	0.90	0.9	1.0	0.83
AD × *AD*	45.6	0.74	0.92	0.93	0.9	0.9	0.77
AD × *DD*	50.3	0.81	0.92	0.90	0.9	1.0	0.83
DD × *AA*	59.5	0.96	1.00	0.90	1.0	1.0	1.00
DD × *AD*	46.9	0.76	0.92	0.95	1.0	0.9	0.87
DD × *DD*	55.3	0.89	0.85	0.98	1.0	1.0	0.92

* Ratio observed taking 61.9% for AA male × DD female as 1.00.
† Ratio expected taking product of weighting factors for AA male × DD female mating and equating to 1.00 by multiplying product by 1.11.

produce only heterozygotes will suffer no disadvantage from this effect and are weighted by the factor 1.00. Matings between like homozygotes, which produce only homozygotes, are at a disadvantage and are weighted by the factor 0.85. This factor comes from analysis of the data presented in Table 3, where it is seen that approximately 87% as many homozygotes as heterozygotes are produced. Matings between homozygote and heterozygote are weighted by the factor 0.92, as only half their zygotes will be homozygotes.

(2) *Mother-fetus incompatibility.* Matings producing only compatible fetuses are given the weighting 1.00 while matings producing equal numbers of compatible and incompatible fetuses are given the weighting factor 0.90. This is an arbitrary value close to the value of 0.87 derived from the data in Table 5. For reasons discussed above it is impossible to get an unbiased estimate of the effect due to incompatibility. Also the model requires the effect of incompatibility to be somewhat less than the effect of heterozygote advantage to meet the observed distribution of breeding efficiencies.

Although the model is developed for the two alleles Tf^A and "Tf^D", the observed distribution of breeding efficiencies was obtained from herds in which Tf^{D1} and Tf^{D2} were segregating. It is necessary to make provision for this in the model. In the mating AD male × AD female there is the possibility of incompatibility between TfAD1 embryos and TfAD2 mothers, TfAD2 embryos and TfAD1 mothers, and TfD1D2 embryos and TfAD1 or TfAD2 mothers. If Tf^{D1} and Tf^{D2} were equally frequent in thsee populations the effect of incompatibility would be less than that from TfAA male × TfDD female (weighted at 0.90), but more than that from TfDD male × TfAD female weighted at 0.95. Thus the mating TfAD × TfAD is given the weighting 0.93. Similarly the mating TfDD male × TfDD female will give rise to a proportion of incompatible zygotes, and is weighted by the factor 0.98.

(3) *Heterozygote fertility.* Both heterozygous bulls and cows are less fertile than their corresponding homozygotes. The exact values for their relative fertility is not clear. The mean unweighted ratio of homozygote: heterozygote bull fertility (Table 6) is 1.091, and the mean unweighted ratio for cow fertility is 1.062 (Table 8). However, cows and bulls coded TfDD will include a proportion of heterozygous TfD1D2 individuals, and it is likely that the ratios for homozygote: heterozygote fertility are underestimates. Homozygote fertility for both cows and bulls is given the weighting factor 1.0, and heterozygote fertility 0.9.

Theoretical values for the relative breeding efficiencies were derived by multiplying the weighting factors for the four effects (heterozygote superiority, incompatibility, bull fertility, cow fertility) and equating the ratio for the best mating, AA male × DD female to 1.00 (Table 9). The ratio of the observed breeding efficiencies (from Table 1) was calculated taking the breeding efficiency of AA male × DD female as 1.00. Comparison of the ratios obtained from the observed breeding efficiencies with those produced by the model showed a good fit.

The model assumes that the effects postulated are independent, which is unlikely to be true. However, it provides a working hypothesis which can be used

as a guide for interpretation of more stringent data as these become available.

Polymorphic equilibrium: The proposed model can be considered satisfactory if in addition to meeting the observed distribution of breeding efficiencies it also results in a balanced polymorphism. This may be examined mathematically, and I am indebted to Dr. Y. Hiraizumi for discussion and assistance with the relevant calculations. The mathematical analysis was limited to consideration of two alleles, Tf^A and a single Tf^D. It consists of a comparison between the distribution of genotypes in any parental generation with that in the first filial generation, using the parameters of the model. From this comparison it is possible to determine whether the model would lead to a balanced polymorphism.

The frequency of each mating in the parental generation is readily calculated from the frequency of each parental genotype. From each mating the expected distribution of progeny after applying weighting factors for heterozygote superiority, incompatibility and male and female heterozygote fertility can be derived (Table 10). The expected distribution of each genotype in the progeny can be determined by summation and is equivalent to:

Frequency of AA in progeny at generation $t = p_t = \Sigma AA/\bar{w}$
Frequency of AD in progeny at generation $t = q_t = \Sigma AD/\bar{w}$
Frequency of DD in progeny at generation $t = 1\text{-}p_t\text{-}q_t = \Sigma DD/\bar{w}$
where $\bar{w} = \Sigma AA + \Sigma AD + \Sigma DD$.

Putting: V_{AA} = relative viability of TfAA progeny = 0.85
V_{DD} = relative viability of TfDD progeny = 0.85
$V_{AD}{}^c$ = relative viability of TfAD progeny in compatible (TfAD) mothers = 1.00

TABLE 10

Expected genotype and mating frequencies in parents at generation t *and progeny in generation* t + 1

Parents at generation t		Progeny at generation $(t+1)$		
♂	♀	AA	AD	DD
AA	$\times AA$	$p_t{}^2V_{AA}F_{AA}f_{AA}$	.....	.....
AA	$\times AD$	$\frac{1}{2}p_tq_tV_{AA}F_{AD}f_{AA}$	$\frac{1}{2}p_tq_tV_{AD}{}^cF_{AD}f_{AA}$	.....
AA	$\times DD$	.....	$p_t(1-p_t-q_t)V_{AD}{}^iF_{DD}f_{AA}$	.....
AD	$\times AA$	$\frac{1}{2}p_tq_tV_{AA}F_{AA}f_{AD}$	$\frac{1}{2}p_tq_tV_{AD}{}^iF_{AA}f_{AD}$	.....
AD	$\times AD$	$\frac{1}{4}q_t{}^2V_{AA}F_{AD}f_{AD}$	$\frac{1}{2}q_t{}^2V_{AD}{}^cF_{AD}f_{AD}$	$\frac{1}{4}q_t{}^2V_{DD}F_{AD}f_{AD}$
AD	$\times DD$	.....	$\frac{1}{2}q_t(1-p_t-q_t)V_{AD}{}^iF_{DD}f_{AD}$	$\frac{1}{2}q_t(1-p_t-q_t)V_{DD}F_{DD}f_{AD}$
DD	$\times AA$	.....	$p_t(1-p_t-q_t)V_{AD}{}^iF_{AA}f_{DD}$	.....
DD	$\times AD$	.....	$\frac{1}{2}q_t(1-p_t-q_t)V_{AD}{}^cF_{AD}f_{DD}$	$\frac{1}{2}q_t(1-p_t-q_t)V_{DD}F_{AD}f_{DD}$
DD	$\times DD$	.....	.....	$(1-p_t-q_t)^2V_{DD}F_{DD}f_{DD}$
		ΣAA	ΣAD	ΣDD

Frequency of parental genotypes TfAA$=p_t$, TfAD$=q_t$, and TfDD$=(1-p_t-q_t)$. For other symbols see text.

$V_{AD}{}^{i}$ = relative viability of TfAD progeny in incompatible (TfAA or TfDD) mothers = 0.90

and: Fertility of TfAA cows = $F_{AA} = 1.0$
Fertility of TfDD cows = $F_{DD} = 1.0$
Fertility of TfAD cows = $F_{AD} = 0.9$
Fertility of TfAA bulls = $f_{AA} = 1.0$
Fertility of TfDD bulls = $f_{DD} = 1.0$
Fertility of TfAD bulls = $f_{AD} = 0.9$

Then:

$$\frac{dp_t}{dt} = \frac{1}{\bar{w}}\,[q_t{}^2(0.1721 + 0.0208p_t) + p_tq_t(0.845 + 0.1p_t) + p_t(0.1p_t{}^2 + 0.75p_t - 0.85)]$$

$$\frac{dq_t}{dt} = \frac{1}{\bar{w}}\,[0.0208q_t{}^3 + q_t{}^2(0.1p_t - 0.37) + q_t(0.1p_t{}^2 - 1.9p_t + 0.005) + 1.8p_t(1 - p_t)]$$

and $$\bar{w} = 0.85 + 0.1p_t - 0.08q_t - 0.1p_t{}^2 - 0.0208q_t{}^2 - 0.1p_tq_t$$

If selection operates on Tf^A and Tf^D it is assumed to be equal. Then at equilibrium the following relationship will hold:

$$\hat{p} + 0.5\hat{q} = 0.5$$

where $\hat{p}$ and $\hat{q}$ are the estimated equilibrium frequencies of p and q.
When $dp_t/dt = 0$, then $0.89q^3 - 26q^2 + 184q - 90 = 0$.

Of the three roots to this equation two are imaginary, and the meaningful root gives:

$\hat{q} = 0.5278$ = equilibrium frequency of TfAD

and hence

$\hat{p} = 0.2361$ = equilibrium frequency of TfAA
$1-\hat{p}-\hat{q} = 0.2361$ = equilibrium frequency of TfDD.

This implies that a population at equilibrium will show an excess of heterozygotes of 5.6% which is in good agreement with the values actually found (Table 4; and ASHTON and FALLON 1962).

The stability of this equilibrium, maintained by heterozygote advantage, can be tested by applying the "characteristic equation" (LOTKA 1956). Let $p_t = \hat{p} + \Delta p_t$, and $q_t = \hat{q} + \Delta q_t$, where Δp_t and Δq_t are slight deviations from the equilibrium values of $\hat{p}$ and $\hat{q}$. Then

$$\frac{d\Delta p_t}{dt} = -0.0024\Delta p_t + 0.3020\Delta q_t + \ldots \text{ (higher order values of } \Delta p_t \text{ and } \Delta q_t)$$

$$\frac{d\Delta q_t}{dt} = 0.00\Delta p_t - 0.7863\Delta q_t + \ldots \text{ (higher order values of } \Delta p_t \text{ and } \Delta q_t).$$

and the characteristic equation for this system is

$$\begin{vmatrix} -0.0024\Delta p_t - \lambda & 0.3920 \\ 0.00 & -0.7863 - \lambda \end{vmatrix} = 0$$

giving $\lambda = -0.0024$ or -0.7863.

Since both values of λ are real and negative the equilibrium very probably is stable, for under these conditions Δp_t and Δq_t approach zero with elapsing time.

It should be noted that the differential equations dp_t/dt and dq_t/dt are more strictly written as finite difference equations, for example, $\Delta p_t + 1$. The arithmetic involved in the solution of such equations is complex, and the approximate but more readily soluble differential equations have been used here to obtain a general solution. A computer program for examining stability of equilibria is currently being written in this department (YASUDA, personal communication). This will permit checking of the equilibrium for other values of p and q.

Selection: A polymorphism maintained by heterosis is usually subject to selective pressures, evidenced by inequality in the frequencies of the genes comprising the polymorphism. The nature of the selective forces acting on cattle transferrins in the feral state will most likely remain conjectural. However, evidence of powerful man-made selection is available from several sources, favors the transferrin D alleles, and is associated with selection for milk yield.

OSTERHOFF (1964) has examined the distribution of transferrin genotypes of female dairy cattle in different age groups. Up to 12 months of age the frequencies of Tf^A and Tf^D were about the same, approximately 0.48, and the frequency of Tf^E about 0.04. Tf^D then showed a progressive rise and Tf^A a progressive fall in frequency with advancing age of the group examined, Tf^D approaching 0.65 and Tf^A 0.25. The frequency of Tf^E also increased slightly approximating 0.1. OSTERHOFF points out that selection for higher milk yield is a continuous process, with the result that the older cows usually are the best producers. Other evidence of the effect of selection for milk yield on transferrin gene frequency comes from the data presented by LARSEN (1961). He examined the transferrin types of the three highest producing and three lowest producing heifers in each of 18 groups in the Danish progeny testing stations in 1959–60. The transferrin gene frequencies in the lowest yielding group were Tf^A 0.731, Tf^D 0.241 and Tf^E 0.028, and in the highest yielding group Tf^A 0.630, Tf^D 0.333 and Tf^E 0.037. Although the difference in gene frequencies was not significant, Tf^D is more frequent in the high producing group.

Selection for Tf^D is also apparent in the available segregation data from the six published sources examined in Tables 3 and 5. Six matings are available for assessing this effect, uncomplicated by heterozygote advantage or differential maternal-fetal incompatibility (Table 11). The relative "survival" of the progeny from these matings can be ranked in the order D > E > A. While it is true that

TABLE 11

Distribution of progeny from certain matings pooled from the six sources quoted in Tables 3 and 5, showing selection favoring $Tf^D > Tf^E > Tf^A$

Mating ♂ × ♀	Progeny genotypes With Tf^A	With Tf^D	With Tf^E
AD × *EE*	1 *AE*	13 *DE*	..
AD × *AD*	154 *AA*	191 *DD*	..
DE × *AA*	..	12 *AD*	8 *AE*
DE × *DE*	..	10 *DD*	7 *EE*
AE × *DD*	11 *AD*		15 *DE*
AE × *AE*	7 *AA*	..	9 *EE*

those results could also be explained by postulating prezygotic selection of D sperm in preference to E or A sperm it seems much more likely that postnatal selection for TfDD cows is the real cause. It must be borne in mind that the progeny which are available for segregation anlysis come mainly from dairy herds, will be mainly females, and will be from different age groups. Such considerations also make it more difficult to measure the extent of the effects shown in Tables 3 and 5.

Direct evidence that milk yield is influenced by transferrin type was obtained by ASHTON, FALLON, and SUTHERLAND (1964) who found that TfDD cows produced an average 45 imperial gallons of milk more than TfAA cows, TfAD cows being intermediate. These results confirmed earlier observations of ASHTON (1960) who examined the contemporary comparisons of bulls, found significant differences and forecast that TfDD cows would yield on average about 50 gallons more milk per lactation than TfAA cows.

While strong man-made selection is affecting the frequency of Tf^D in dairy herds the frequency of Tf^A is still high, and in some cases higher than Tf^D (ASHTON 1958b; MARKUS, KOVACS, and FÉSÜS 1964). The reasons for this are not known, but it seems an inescapable conclusion that Tf^A is at a considerable advantage, as yet undetermined.

Tf^E is usually present at low frequency in most European breeds. Its frequency is always higher in these breeds when they are located in the tropics. Tf^E is also frequent in zebu breeds (ASHTON and LAMPKIN 1965), and it seems likely that the selective advantage maintaining this allele is somehow associated with tolerance to hotter climates. Unpublished observations revealed that sweating rate of TfAE and TfDE beef cattle is greater than of animals lacking Tf^E. Sweat and milk glands have a common embryological origin, and a common mechanism may link the advantages of Tf^D and Tf^E. If Tf^E also is maintained by heterozygote advantage, the necessary information will only come from studies of populations with a high frequency of this allele. So far the information available about fertility of Tf^E cows and bulls and segregation of Tf^E genotypes is too scanty to warrant consideration. Similar comments apply to the transferrin alleles Tf^B, Tf^F, and Tf^G found in zebu cattle.

Conclusion: The data summarized here show significant effects for the four aspects of fertility somehow affected by transferrin type. While maternal-fetal incompatibility is reasonably well established for other antigenic systems, particularly the Rh and ABO blood groups in man, it is not at all clear why heterozygous fetuses should be more viable *in utero* than homozygotes, and quite conjectural as to how heterozygous parents can be overall less fertile than homozygotes. Because of these gaps in our knowledge the title to this paper requires a question mark. Unequivocal demonstration of a polymorphism balanced in the complex manner described will require more stringent data than are now available.

SUMMARY

Fertility in cattle is influenced by four effects of the serum transferrin locus. These are (1) heterozygote advantage *in utero*, (2) maternal-fetal incompati-

bility, (3) poorer fertility of heterozygous bulls, and (4) poorer fertility of heterozygous cows. Estimates of these effects were derived from published data. A model with these parameters produced the observed distribution of breeding efficiencies. The model leads to an apparently stable equilibrium for the two alleles considered, with an estimated population excess of heterozygotes very close to that observed.

LITERATURE CITED

ALLISON, A. C., 1956 Population genetics of abnormal human haemoglobins. Acta Genet. Statist. Med. **6**: 430–434.

ASHTON, G. C., 1958a β-globulin polymorphism and early foetal mortality in cattle. Nature **183**: 404–405. —— 1958b Polymorphism in the serum proteins of mammals, with particular reference to cattle. Thesis, University of Liverpool. —— 1960 β-globulin polymorphism and economic factors in dairy cattle. J. Agric. Sci. **54**: 321–328. —— 1961 β-globulin type and fertility in artificially bred dairy cattle. J. Reprod. Fertil. **2**: 117–129. —— 1963 Polymorphism in the serum post-albumins of cattle. Nature **198**: 1117–1118. —— 1965 Serum transferrin-D alleles in Australian cattle. Aust. J. Biol. Sci. **18**: 665–670.

ASHTON, G. C., and G. R. FALLON, 1962 β-globulin type, fertility and embryonic mortality in cattle. J. Reprod. Fertil. **3**: 93–104.

ASHTON, G. C., G. R. FALLON, and D. N. SUTHERLAND, 1964 Transferrin (β-globulin) type and milk and butterfat production in dairy cows. J. Agric. Sci. **62**: 27–34.

ASHTON, G. C., and G. H. LAMPKIN, 1965 Transferrin and post-albumin polymorphism in East African cattle. Genet. Res. **6**: 209–215.

BRUMMERSTEDT-HANSSEN, E., J. MOUSTGAARD, and I. MØLLER, 1963 Serum transferrin polymorphism in Danish cattle breeds. Roy. Vet. Agric. Coll. Copenhagen, Yearbook, 13–26.

DATTA, S. P., 1963 Immunogenetic studies of cattle transferrins and blood groups. Thesis, University of Wisconsin.

FISHER, R. A., 1930 *The Genetical Theory of Natural Selection.* Clarendon Press, Oxford.

FORD, E. B., 1964 *Ecological Genetics.* Wiley, New York.

GAHNE, B., 1961 Studies of transferrins in serum and milk of Swedish cattle. Anim. Prod. **3**: 135–144.

GALL, G. A. E., and R. T. BERG, 1964 Studies of the inheritance of bovine serum transferrins. Anim. Prod. **6**: 107–117.

HICKMAN, C. G., and H. O. DUNN, 1961 Differences in percentage of non-returns to service between transferrin types of bulls. Can. J. Genet. Cytol. **3**: 391–395.

KRISTJANSSON, F. K., 1962 Recent research in serum protein polymorphisms of livestock. Proc. 8th Annual Blood Group Conference, Ljubljana, Yugoslavia. Mimeographed proceedings.

LARSEN, H., 1961 Investigations concerning the possible influence of serum β-globulins, haemoglobins and β-lactoglobulins upon yielding ability of cattle. Inst. F. Sterilitetsforskning, Aarsberetning. pp. 125–134.

LOTKA, A. J., 1956 *Elements of Mathematical Biology*, p. 60. Dover, New York.

MÁRKUS, J., G. KOVÁCS, and L. FÉSÜS, 1964 The frequency of genes controlling serum β-globulin types in Hungarian spotted cattle. Acta Vet. **14**: 437–442.

MORTON, N. E., and C. S. CHUNG, 1959 Are the MN blood groups maintained by selection? Amer. J. Human Genet. **11**: 237–251.

New Zealand Dairy Production and Marketing Board, 1963 Farm Production Report No. 39, p. 54.

OSTERHOFF, D. R., 1964 Recent research on biochemical polymorphism in livestock. J. S. Afr. Vet. Med. Ass. **35**: 363–380.

ROBERTSON, A., 1965 The interpretation of segregation ratios in domestic animal populations. Anim. Prod. **7**: (In press).

SHEPPARD, P. M., 1958 *Natural Selection and Heredity*. Hutchison, London.

SMITH, C. A. B., 1961 Statistical methods and theory. p. 140. *Recent Advances in Human Genetics*. Edited by L. S. PENROSE. Little, Brown, Boston.

SNEDECOR, G. W., 1956 *Statistical Methods*. 5th ed., p. 213. Iowa State College Press., Ames.

WOOLF, B., 1955 On estimating the relation between blood group and disease. Eugenics **19**: 251–253.

SECTION "B": OTHER GENETIC SYSTEMS IN CATTLE.

Paper No.	Title	Describes: -
12	Lack of "Slow-Alpha" proteins in some Guernsey cattle. (1958)	-first account of polymorphism in $S\alpha_2$ proteins of any species.
13	Polymorphism in serum post-albumins of cattle. (1963)	-first account of post-albumin polymorphism in a species other than man.
14	Serum albumin and transferrin polymorphism in East African cattle. (1965)	-first description of serum albumin polymorphism in mammals (and description of a further transferrin allele).
15	Serum albumin polymorphism in cattle. (1964)	-detailed account of serum albumin polymorphism.
16	A genetic mechanism for "thread protein" polymorphism in cattle. (1958)	-first account of inheritance of serum amylases.
17	Serum amylase (thread protein) polymorphism in cattle. (1965)	-recognition of "thread proteins" as serum amylases.
18	Stability of tick resistance in cattle: its correlation with various genetic characteristics. (1966)	-a possible association between amylase type and susceptibility to tick infestations.
19	Distribution of transferrin, post-albumin, amylase and haemoglobin genotypes in Droughtmaster cattle. (1966)	-a search for evidence of heterozygote superiority in cattle serum polymorphisms.

(*Reprinted from Nature, Vol.* 182, *pp.* 193–194, *July* 19, 1958)

Lack of 'Slow-Alpha' Proteins in some Guernsey Cattle

Out of about 2,500 cattle sera examined during the past two years by starch gel electrophoresis[1], two individuals were found which lacked a slow alpha (Sα) protein zone (Fig. 1). Both animals were Guernsey cattle, one a five-year-old cow and the other her twelve-month-old heifer calf. This suggests that the condition is inherited. The herd from which the cow originated was examined for the absence of Sα-proteins. The part of the pedigree which is relevant is shown in Fig. 2.

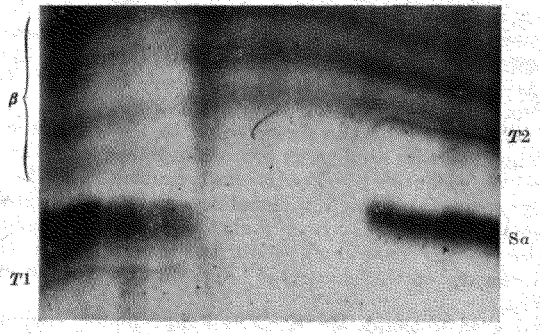


Fig. 1. Part of the anodic side of a starch-gel electropherogram carrying three cattle sera, the central one lacking Sα-proteins. β, β_2-globulins; $T1$ and $T2$, thread proteins

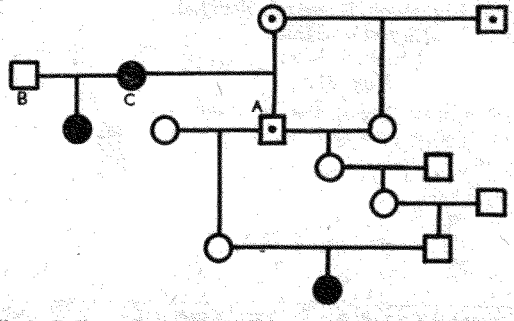


Fig. 2. Pedigree of part of a Guernsey herd in which some animals lack Sα-proteins. Solid circles denote affected females. Symbols with dots indicate animals which were not available

It seems likely that the Sα-protein (or proteins) is controlled by a pair of allelomorphic genes, $S\alpha^A$ and $S\alpha^O$. The genotypes $S\alpha^A/S\alpha^A$ and $S\alpha^A/S\alpha^O$ would then give phenotypes with Sα-proteins, while the genotype $S\alpha^O/S\alpha^O$ would lack them.

The relatively high frequency of $S\alpha^O$ in this herd seems to be due to a bull (unfortunately dead) which must have been homozygous for this allele. This particular bull (*A* in Fig. 2) was the result of a cross between two animals which had the same dam.

Insufficient information is available to assess the frequency of $S\alpha^O$. However, the bull *B* (Fig. 2), presumably $S\alpha^A/S\alpha^O$, is at present standing at a cattle breeding centre and is used extensively. No further examples of individuals lacking Sα-proteins were found among his progeny from fourteen matings picked at random from other herds.

The animals lacking Sα-proteins were in good health and did not show any signs of abnormalities. In fact, the five-year-old cow (*C*, Fig. 2) gave the best individual milk yield of cows in milk-recording Guernsey herds in Staffordshire for two successive years. Each of the bull *A*'s five daughters in the herd has shown a milk yield above average in each lactation.

The nature of the Sα-proteins in the cow is not known, but an analogous protein in humans has been shown recently by Poulik and Smithies[2] to be a glycoprotein of high molecular weight. A protein zone of this type has been found in all the mammalian sera so far examined, with the exception of the Guernsey cattle shown in Fig. 2 and two horse sera.

I wish to thank Mr. K. O'Sullivan, deputy regional veterinary officer of the Milk Marketing Board, for his generous co-operation.

G. C. Ashton

Farm Livestock Research Station,
Lilystone Hall,
Stock, Essex.
May 21.

[1] Ashton, G. C., *Nature*, **180**, 917 (1957).
[2] Poulik, M. D., and Smithies, O., *Biochem. J.*, **68**, 636 (1958).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

COMMONWEALTH OF AUSTRALIA

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

(*Reprinted from Nature, Vol.* 198, *No.* 4885, *pp.* 1117–1118, *June* 15, 1963)

(13)

Polymorphism in the Serum Post-albumins of Cattle

THREE polymorphic serum protein systems have been described so far in cattle, namely, transferrins[1–3], thread proteins[4], and slow-α-globulins[5]. A fourth polymorphic system, involving the post-albumins, has been found and is the subject of this communication.

The polymorphic post-albumins are revealed by starch-gel electrophoresis in a modified discontinuous borate electrolyte, *tris*–citric buffer system[6], using the horizontal technique of Smithies[7]. With this system[8] the electrolyte in the electrode compartments is a solution containing 1·2 g of lithium hydroxide and 11·8 g of boric acid per litre, *p*H 8·2. The gel is prepared with hydrolysed starch from the Connaught Laboratories, Toronto, Canada, using a buffer made by adding 90 vols. of a solution containing 1·6 g citric acid and 6·2 g *tris*(hydroxymethyl)aminomethane per litre, to 10 vols. of electrolyte. During electrophoresis the gels are water-cooled to remove the Joule's heat, and with an applied voltage of 14–15 V/cm length of gel the trailing edge of the albumin zone migrates 10 cm in about 2 h. To achieve good resolution of the post-albumin proteins the albumin zone must be compact. Best results have been achieved when the albumin zone is about 5–7 mm wide with the conditions described.

The polymorphic proteins appear immediately behind the trailing edge of the albumin zone, and according to the nomenclature proposed by Smithies[7] for human serum can be described as 'post-albumins'. Three phenotypes have been seen (Fig. 1). In one, two fast migrating zones are present in the post-albumin position (fast type). In the second two slower-migrating post-albumin zones are seen (slow type). In the third (slow–fast type), both pairs of post-albumin zones appear to be present, although the faster, fainter zone of each pair is not always easy to see. By analogy with other polymorphic serum protein systems, particularly the transferrins[8,9], it seems likely that this post-albumin system in cattle is controlled by two co-dominant alleles. Each allele would give rise to two zones in starch gel, a faster faintly staining zone, and a slower, more intensely staining zone. Designating the locus by the symbol *Pa*, the two alleles could be coded Pa^F (fast) and Pa^S (slow). The three genotypes Pa^F/Pa^F, Pa^S/Pa^S, and Pa^S/Pa^F would correspond with the three observed pheno-

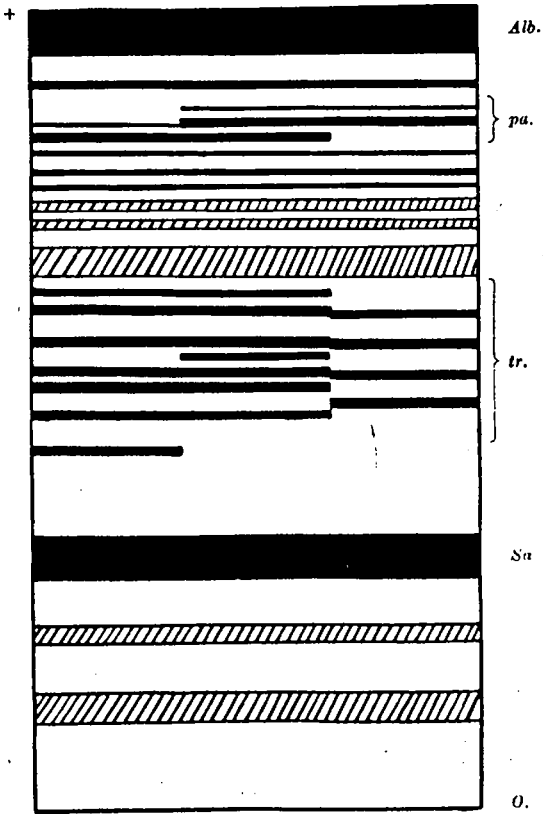


Fig. 1. Diagram of a stained gel showing the three post-albumin phenotypes (left to right), slow, slow–fast, and fast. The corresponding transferrin types are *A/E*, *A/F*, and *D/D*. The cross-hatched areas represent diffuse zones. The diagram is to scale, the distance on the gel from the origin to the trailing edge of the albumin zone was 10·05 cm. *Alb.*, albumins; *pa.*, polymorphic post-albumins; *tr.*, transferrins; *Sa*, slow-*a*; *O.*, point of sample insertion

types fast, slow and slow–fast. Limited mating data support this hypothesis.

The distribution of post-albumin phenotypes in 221 beef cattle from the herd at the National Cattle Breeding Station is shown in Table 1. In the British breeds (derived from repetitive crossing of Shorthorn and Hereford cattle) the slow phenotypes predominate. In the Afrikaner × British cross-breds (F_2 generation Afrikaner × Shorthorn and Afrikaner × Hereford cattle) the fast and slow–fast types are more frequent. These types are also frequent in the Brahman cross-breds (F_2 generation Brahman × Shorthorn and Brahman × Hereford cattle) and in the grade Brahmans ($\frac{3}{4}$–$\frac{7}{8}$ Brahman) graded up

Table 1. Distribution of Post-albumin Phenotypes in Various Cross-breeds of Beef Cattle at the National Cattle Breeding Station

Breed	No. of Animals	Phenotype slow	slow-fast	fast
British	58	43	8	7
Afrikaner cross-breds	89	26	32	31
Brahman cross-breds	57	22	24	11
Grade Brahmans	17	3	11	3

from Brahman × Shorthorn crosses. In a sample of 57 Jersey and Australian Illawarra Shorthorn dairy cattle only the slow phenotype was observed. There is clearly a marked breed difference in the gene frequencies in this polymorphism.

In this work there was no indication of linkage between the transferrin, thread protein or post-albumin loci.

A more detailed account of this investigation will appear elsewhere. I thank the Officer-in-Charge, National Cattle Breeding Station, 'Belmont', Rockhampton, Queensland, for access to the cross-bred beef cattle, Mr. A. Packham for the samples of dairy cattle blood, and Mr. M. N. Dennis for assistance.

G. C. Ashton

C.S.I.R.O.
Cattle Research Laboratory,
Division of Animal Genetics,
Rockhampton, Queensland, Australia.

[1] Ashton, G. C., *Nature*, **182**, 370 (1958).
[2] Smithies, O., and Hickman, C. G., *Genetics*, **43**, 374 (1958).
[3] Ashton, G. C., *Nature*, **184**, 1135 (1959).
[4] Ashton, G. C., *Nature*, **182**, 65 (1958).
[5] Ashton, G. C., *Nature*, **182**, 193 (1958).
[6] Poulik, M. D., *Nature*, **180**, 147 (1957).
[7] Smithies, O., *Biochem. J.*, **61**, 629 (1955).
[8] Ashton, G. C., and Braden, A. W. H., *Austral. J. Biol. Sci.*, **14**, 248 (1961).
[9] Ashton, G. C., and Ferguson, K. A., *Genet. Res.* (in the press).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(14.)

COMMONWEALTH OF AUSTRALIA
COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

(*Reprinted from Nature, Vol.* 205, *No.* 4967, *pp.* 209–210, *January* 9, 1965)

Serum Albumin and Transferrin Polymorphism in East African Cattle

A SURVEY of the serum proteins of several breeds of East African cattle by starch-gel electrophoresis has recently been carried out. During this survey, a new transferrin phenotype was seen and a previously undescribed variation in the serum albumin, presumably representing serum albumin polymorphism, was found.

The starch-gel procedure used was essentially that described by Ashton and Braden[1] for mice serum proteins and by Ashton and Ferguson[2] for sheep. In this system the electrolyte was composed of 2·2 g lithium hydroxide monohydrate (53–55 per cent LiOH) and 11·8 g boric acid per litre of solution. The gel was prepared from hydrolysed starch (Connaught Laboratories, Toronto, Canada) at the recommended strength, and the buffer consisted of 450 ml. of a solution containing 1·6 g *tris* (hydroxymethyl)-aminomethane, and 7·8 g citric acid per litre, to which was added 50 ml. of the electrolyte. The hot gel solution was poured into a tray containing six adjacent compartments, each 0·3 × 4 × 25 cm internally, and the electrolyte was connected to the gel by a thin plastic sponge (Wettex, Aust. Pty., Ltd., Sydney, Australia) which overlapped the gel by 4 cm at either end. The samples were inserted 8 cm from the cathode end of the gel, on very thin filter paper (Ekwip Industrial Equipment Pty., Ltd., Sydney, Australia). Either three or four samples per compartment were inserted, one a reference sample, and the gel was covered with polythene film to avoid evaporation during electrophoresis.

With this system, when an initial current of 100 m.amp per gel was passed, optimal resolution was obtained after about 2 h. The albumin zone, which should be narrow (2–3 mm), had then migrated about 9–10 cm, and the brown zone of discontinuity about 13–14 cm. This system gives excellent resolution of transferrin[4], post-albumin[3], and albumin polymorphism.

The serum samples examined were from Boran, Sahiwal, Nganda, Teso, Ankole and Tanganyika shorthorned Zebu cattle. Full details of the gene frequencies and other information will be reported elsewhere. From the total of about 1,100 samples representing the different types of

cattle, three Borans out of a group of approximately 300 from the East African Veterinary Research Organization. Genetics herd showed a previously undescribed transferrin phenotype (Fig. 1). The three animals showing this type were members of the same family, and it seems probable that they represent the heterozygote of *Tf*^{F} (ref. 5) and a 'new' allele which, following the pattern previously adopted[2,5], will be coded provisionally with the next available letter, *Tf*^{G}. The new phenotype is accordingly Tf G/F. The homozygote Tf G/G will presumably show the usual four zones[6], and the slowest of these will align with the second fastest zone of Tf A/A (Fig. 1). The phenotypes of other heterozygotes of *Tf*^{G} may be forecast by the diagrammatic approach used for *Tf*^{B} and *Tf*^{F} in Zebu cattle[5].

Serum albumin polymorphism is shown in Fig. 2. Although it can be detected in the described system in gels with undiluted serum samples inserted, it is revealed more readily when the serum is diluted to one part in six with water before electrophoresis. Five albumin phenotypes have been recognized, three of which appear to be common and two rare. The three common types have been coded A, AB and B, and the two rarer types AC and BC (Fig. 2). The three common types occurred in

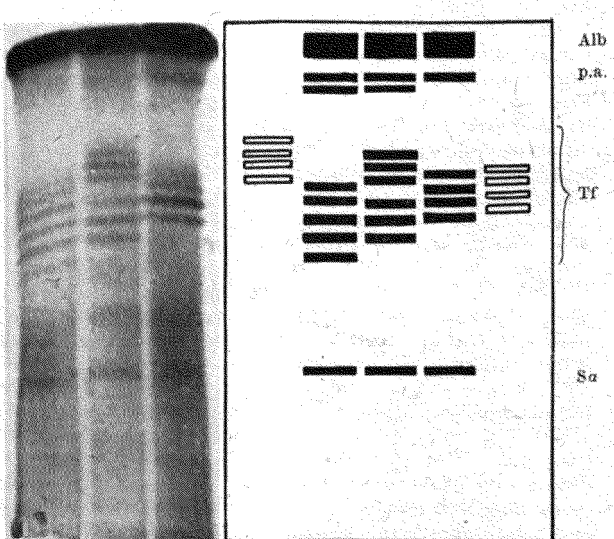


Fig. 1. Photograph and corresponding diagram of a gel carrying three serum samples, showing (left to right) transferrin types B/E, G/F and B/B. The diagram also shows the expected position of the postulated homozygote G/G, and the relative position of an A/A phenotype. Alb, albumin; p.a., post-albumins; Tf, transferrins; Sα, slow-α globulins

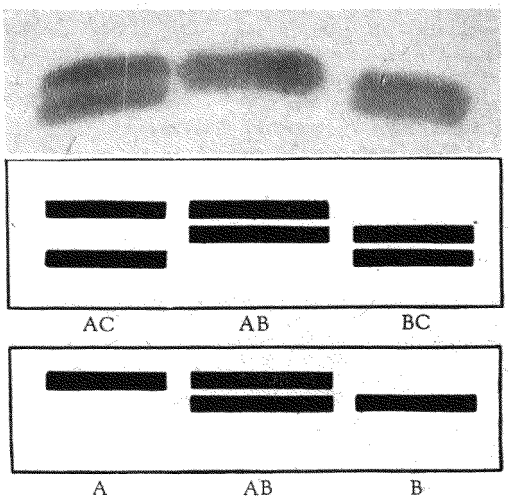


Fig. 2. Photograph and corresponding diagram (centre) of the albumin region of a gel carrying three diluted serum samples, showing (left to right) the albumin phenotypes AC, AB and BC. The lower diagram shows the three common phenotypes A, AB and B

all the breeds examined, but the two rarer types were found only in the Tanganyika shorthorned Zebu. However, only the latter and the Ankole samples were examined in detail for albumin polymorphism, and the presence of the AC and BC phenotypes in the other breeds is not excluded.

The 74 Ankole sera which were examined in detail included 17 A, 29 AB and 28 B phenotypes, and among 83 Tanganyika shorthorned Zebu sera there were 16 A, 25 AB and 42 B types. In both breeds the distribution of phenotypes was compatible with Hardy–Weinberg distribution for a two-allele, three-genotype system. It seems probable that the albumin variants described reflect serum albumin polymorphism. It is therefore proposed that the locus symbol for this system is *Alb* and the two common alleles, Alb^A and Alb^B. The three common phenotypes found may then prove to be Alb A/A, Alb A/B, and Alb B/B. The two rarer phenotypes BC and AC may represent the products of Alb^A and Alb^B, with a third allele, Alb^C. So far, no mating data are available to test this hypothesis. European and other breeds of cattle are to be examined for this polymorphism.

McIndoe[7] has described serum albumin polymorphism in the domestic fowl. Two serum albumins have also been described in some of the members of a few human families. Serum albumin polymorphism may well be a widespread phenomenon.

We thank the director and staff of the East African Veterinary Research Organization for facilities and for the collection of samples.

G. C. ASHTON

C.S.I.R.O. Cattle Research Laboratory,
Rockhampton, Queensland,
Australia.

G. H. LAMPKIN

East African Veterinary Research Organization,
Muguga, P.O. Box 32,
Kikuyu, Kenya.

[1] Ashton, G. C., and Braden, A. E., *Austral. J. Biol. Sci.*, **14**, 248 (1961).
[2] Ashton, G. C., and Ferguson, K. A., *Genet. Res.*, **4**, 240 (1963).
[3] Ashton, G. C., *Nature*, **197**, 1117 (1963).
[4] Ashton, G. C., *Nature*, **182**, 65 (1958).
[5] Ashton, G. C., *Nature*, **184**, 1135 (1959).
[6] Ashton, G. C., and McDougal, E. I., *Nature*, **182**, 945 (1958).
[7] McIndoe, W. M., *Nature*, **195**, 353 (1962).
[8] Knedel, M., *Clin. chim. Acta*, **3**, 71 (1958).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Reprint from GENETICS, Vol. 50, No. 6, December, 1964. *Printed in U.S.A.*

SERUM ALBUMIN POLYMORPHISM IN CATTLE

G. C. ASHTON[1]

C.S.I.R.O. Division of Animal Genetics, Cattle Research Laboratory, Rockhampton, Queensland, Australia

Received July 17, 1964

FIVE cattle serum albumin phenotypes resolved by starch gel electrophoresis have been described briefly by ASHTON and LAMPKIN (1964). It was suggested that the variation represented serum albumin polymorphism controlled by a locus with three alleles. Further observations on albumin phenotypes have been made on British and Zebu beef and dairy cattle in Australia and are the subject of this report.

MATERIALS AND METHODS

Serum samples examined: Serum samples were obtained from bulls, dams and offspring from the crossbreeding experiment at the National Cattle Breeding Station, "Belmont", Rockhampton, Queensland. The cattle sampled were F_1 Brahman × Shorthorn, Brahman × Hereford, Africander × Shorthorn, Africander × Hereford, and Shorthorn × Hereford, and the F_2 progeny from crosses within the F_1 breed groups. Some purebred beef Shorthorns from another property were also examined.

Serum samples from dairy cattle of the Australian Illawarra Shorthorn, Jersey, Guernsey and Friesian breeds were examined, and also a number of samples from the descendants of Sahiwal and Red Sindhi cattle imported into Australia from Pakistan.

Starch gel electrophoresis: The original detection of serum albumin polymorphism in cattle was made with whole serum in a discontinuous Tris-citric system at pH 8.6 (ASHTON and LAMPKIN 1964). Typing was found to be easier if the serum was diluted with five volumes of water before electrophoresis. With this system each phenotype gave either one or two discrete, well defined zones (Figure 1). Further work with a variety of continuous and discontinuous buffer systems has shown that the resolution of the albumin phenotypes is increased by lowering the pH of the gel buffer (see RESULTS).

In the chosen procedure the electrolyte and gel buffer have the same composition and contain 0.84 g citric acid and 1.0 g Tris(hydroxymethyl)aminomethane per litre of solution. The pH of this solution approximates 5.6 and is not critical. The gels were prepared from hydrolysed starch (Connaught Laboratories, Toronto, Canada) at the concentration recommended by the manufacturers, by the procedure described by KRISTJANSSON (1963). The electrophoresis was performed in a horizontal plane in an apparatus described previously (ASHTON and LAMPKIN 1964). With an applied current of 2.7 ma per cm width of gel, the albumin zones moved approximately 5 cm in 2½ hours.

RESULTS

Phenotypes: Three phenotypes, Alb A, Alb AB, and Alb B were found in a herd of Sahiwal cattle in Kenya. Three phenotypes, apparently the same, have been found in the descendants of Sahiwal cattle imported into Australia. It has not been possible because of quarantine restrictions to compare Kenyan and

[1] Present address: Department of Genetics, University of Hawaii, Honolulu 14.

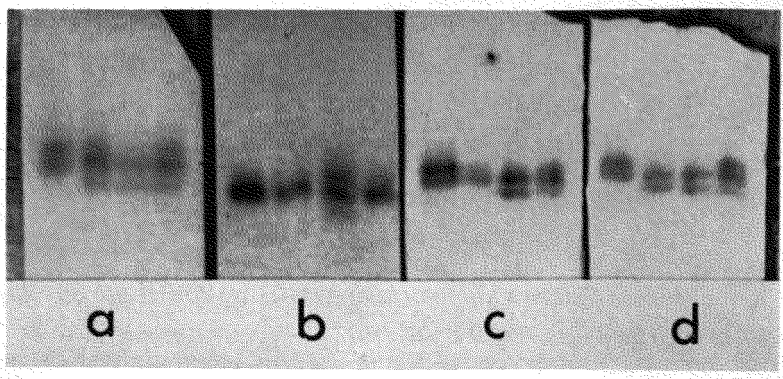


FIGURE 1.—Four gels each with four serum samples showing albumin phenotypes. From left to right, Gel (a) stored sera; Alb A, Alb AB, Alb B, Alb AB. Gel (b) Alb A, fresh serum; Alb A, fresh serum; Alb AB, stored serum; Alb A, fresh serum. Gel (c) Alb A, stored serum; Alb A, fresh serum; Alb B, stored serum; Alb AB, stored serum. Gel (d) stored sera; Alb A, Alb B, Alb B, Alb AB. The anodic end of the gel is at the top of the photograph.

Australian sera directly, but it seems very probable that the three phenotypes seen in Australian Sahiwals correspond with those seen in Kenyan Sahiwals. Accordingly the three phenotypes seen in Australian cattle have been called Alb A, Alb AB and Alb B also. The rare Alb AC and Alb BC phenotypes seen in Tanganyikan Shorthorned Zebu cattle have not yet been seen in Australia.

Careful distinction must be made between the appearance of the starch gel phenotypes obtained by the electrophoresis of fresh sera and their appearance after storage of the sera. With fresh sera at pH 8.2 the Alb A and Alb B phenotypes each show a single zone, while Alb AB shows both these zones. As the pH of the buffer system is lowered each of these zones splits into two, producing a moderately staining faster zone and an intensely staining slower zone. At pH 5.6 these zones are well resolved (Figures 1 and 2). This phenomenon is similar

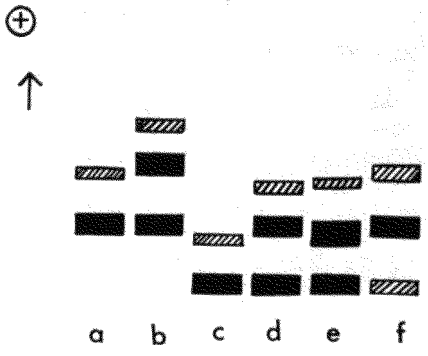


FIGURE 2.—Diagram showing appearance of albumin phenotypes in cattle (a) Alb A from fresh serum, (b) Alb A from stored serum, (c) Alb B from fresh serum, (d) Alb B from stored serum, (e) Alb AB from fresh serum, (f) Alb AB from stored serum. Direction of migration towards anode shown by arrow.

to that described by McINDOE (1962) for serum albumin phenotypes in the domestic fowl.

With serum which has stood on the bench for a few days, or has been stored at −17°C for some months, the appearance of the phenotypes is altered. Alb A and Alb B then show three zones, namely a fast moderately staining zone and two slower intensely staining zones (Figures 1 and 2). Alb AB also shows three zones which stain moderately, intensely, and moderately in decreasing order of mobility.

It will be obvious that care is needed in the interpretation of albumin phenotypes from cattle serum. Alb A is unlikely to be confused with the other phenotypes, but it is difficult to distinguish Alb AB from fresh serum and Alb B from stored serum. Alb AB and Alb B from stored serum are readily distinguished by the differences in relative staining intensity of the three zones and the slight difference in mobility of the fastest zone in the two phenotypes.

Mating results: The distribution of offspring from 460 matings from the National Cattle Breeding Station is shown in Table 1. The type of offspring obtained from a given cross is consistent with the suggestion advanced previously (ASHTON and LAMPKIN 1964) that the three albumin phenotypes Alb A, Alb AB and Alb B represent a polymorphism controlled by two codominant autosomal alleles Alb^A and Alb^B. The genotype Alb^A/Alb^A is thus represented by phenotype Alb A, Alb^A/Alb^B by phenotype Alb AB, and Alb^B/Alb^B by phenotype Alb B.

Breed distribution of albumin types: Alb^B has not been found in dairy and beef cattle of the common European breeds. It was absent in 39 Guernsey cows from one herd, 282 Jersey cows from five herds, and 81 Australian Illawarra Shorthorn cows from two herds. It was not seen in a survey of 128 bulls from artificial breeding centres covering the common dairy breeds, including Friesian. None of the F_1, F_2, or F_3 progeny of beef Shorthorn × Hereford matings at the National Cattle Breeding Station have shown Alb^B, and it was absent from 31 beef Shorthorn steers. Further representative sampling will be necessary to ensure that Alb^B is exclusive to Zebu cattle, but present indications are that it is.

The Brahman crossbred and Africander crossbred cattle on "Belmont" have

TABLE 1

Distribution of progeny phenotypes (observed/expected) from various mating classes and corresponding probability values of χ^2 for the observed/expected deviations

Matings (Alb phenotypes)		Progeny phenotypes			
Male	Female	A	AB	B	P
A	AA	171/171	.....	.....	.....
A	AB	35/44.5	54/44.5	.....	<0.05
AB	A	27/22.0	17/22.0	.....	<0.30
AB	AB	44/37.25	78/74.5	27/37.25	<0.30
AB	B	.....	1/1	1/1	.....
B	AB	.....	2/1.5	1/1.5	.....
B	B	.....	.....	2/2	.....

been derived from a small number of imported Brahman and Africander bulls, and so no valid estimate of gene frequencies in the two parental breeds can be obtained. The same applies to the Sindhi and Sahiwal crossbreds available for examination from the F. D. McMaster Field Station. The high frequency of Alb^B in their progeny, however, shows that this allele was present in at least some of the bulls of each of the four Zebu breeds introduced into Australia.

DISCUSSION

Isolated cases of "double albumins" have been described in humans (EARLE, HUTT, SCHMID and GITTEN 1959) and a genetical basis has been ascribed to the phenomenon. Polymorphism, according to FORD's (1940) definition, of serum albumins was first described by MCINDOE (1962) in the serum of the domestic fowl. STORMONT and SUZUKI (1963) have found albumin polymorphism in the serum of the horse and ASHTON and LAMPKIN (1964) in the serum of cattle. A double albumin has been found in a species of chipmunk by JOHNSON and WICKS (1959), and in a species of Amphibolurus lizard by KIRK and KIRSCH (personal communication). Serum albumin variation has been noted by BECKMAN, CONTERIO and MAINARDI (1963) in certain bird species and their hybrids. It seems likely that albumin polymorphism will prove widespread, as further species and breeds are examined.

In each of the species examined for which mating data are available the genetic hypothesis suggested is autosomal multiple allelism. The types of offspring obtained for each type of mating in the data reported in Table 1 are consistent with such a general hypothesis. The distribution of offspring from the mating *Alb A* male × *Alb AB* female however departs significantly ($P < 0.05$) from that expected from such a hypothesis. It seems unlikely that the two-allele theory advanced to explain these results is incorrect, in view of the results from other species exhibiting albumin polymorphism. Further, the mechanism of multiple allelism is well authenticated in several other serum protein polymorphic systems. The significant deviation from normal expectation suggests that the segregation ratios are aberrant rather than that the two-allele hypothesis is incorrect. In the aberrant mating there is an excess of offspring of the same genotype as the mother. A similar but not significant excess is seen in the reciprocal mating *Alb AB* male × *Alb A* female. These observations if confirmed by further mating data would imply differential viability of the genotypes *in utero*, and would warrant further investigation.

The reason for the very low frequency or complete absence of Alb^B in British breeds of cattle in Australia, compared with its apparently relatively high frequency in African and Indian breeds, is not known. The retention of Alb^B in Zebu cattle implies a selective advantage of this allele at least in the habitat in which Zebus are normally found, but the nature of the advantage is speculative. If Alb^B is found not to occur in European cattle this allele will be a useful marker for assessing the percentage of Zebu in herds in which European × Zebu crossing is practiced.

The AC and BC phenotypes found in Tanganyika shorthorned Zebu cattle were not seen in the present survey, and no mating data are yet available for these types. In the absence of contrary information, it is assumed that these phenotypes represent the genotypes Alb^A/Alb^C and Alb^B/Alb^C, the postulated allele Alb^C producing an albumin complex migrating more slowly than that from Alb^B. It has not been possible to determine whether the albumin produced by the postulated Alb^C also splits into multiple zones at pH 5.6.

The structural difference between the albumins produced by Alb^A and Alb^B has not been determined. The ease with which bovine serum albumin can be isolated and purified should make it a suitable material for studies similar to those of INGRAM (1957) and others on human hemoglobins, although the formation of multiple zones in starch gel will need to be considered. The presence of two zones per allele in fresh serum is reminiscent of the products of transferrin alleles in many species (ASHTON and MCDOUGALL 1958; ASHTON and BRADEN 1961), and of double zones from post-albumin alleles in cattle (ASHTON 1963). The reason for this double or multiple zoning remains obscure but is apparently a general phenomenon of serum proteins and not unique to transferrins. The change in phenotype on storage is possibly due to enzymic action. It is superficially similar to the action of bacterial neuraminidase on serum transferrins and other sialoproteins. However, it differs from these reactions in that the new protein formed during storage is faster in mobility than the original in the case of both Alb A and Alb B phenotypes (Figure 1). In all species examined removal of sialic acid from transferrin phenotypes has resulted in a decreased net charge and a corresponding decrease in mobility. The storage changes affecting the albumin phenotypes may result in dissociation of albumin polymers rather than increase in net charge due to removal of substituents.

STORMONT and SUZUKI (1963) found that the variation in albumin phenotypes in horses was paralleled by variation in postalbumin phenotypes. This is not the case in cattle, where the albumin and postalbumin (ASHTON 1963) genes segregate independently, as also do the transferrins.

Thanks are due to MR. J. F. KENNEDY for access to cattle at the National Cattle Breeding Station, "Belmont", Rockhampton, Queensland, and to MR. R. W. HEWETSON of C.S.I.R.O., Division of Animal Genetics, Wollongbar, New South Wales, for supplying blood samples.

SUMMARY

The results of 460 matings in a beef cattle herd show that albumin polymorphism in cattle is controlled by two autosomal codominant alleles Alb^A and Alb^B. While Alb^B is frequent in Zebu beef and dairy cattle it is apparently absent in European cattle. The appearance of the phenotypes changes on storage and at acid pH.

LITERATURE CITED

ASHTON, G. C., 1963 Polymorphism in the serum postalbumins of cattle. Nature 198: 1117–1118.

ASHTON, G. C., and A. W. H. BRADEN, 1961 Serum β-globulin polymorphism in mice. Australian J. Biol. Sci. **14**: 248–253.

ASHTON, G. C., and G. H. LAMPKIN, 1964 Serum albumin and transferrin polymorphism in East African cattle. Nature (in press).

ASHTON, G. C., and E. I. MCDOUGALL, 1958 Beta-globulin polymorphism in cattle, sheep, and goats. Nature **182**: 945–946.

BECKMAN, L., F. CONTERIO, and D. MAINARDI, 1963 Serum protein variations in bird species and hybrids. Rutgers Univ., Serol. Mus. Bull. **29**: 5–8.

EARLE, D. P., M. P. HUTT, K. SCHMID, and D. GITLIN, 1959 Observations on double albumin A genetically transmitted serum protein anomaly. J. Clin. Invest. **38**: 1412–1420.

FORD, E. B., 1940 Polymorphism and taxonomy. pp. 493–513. *The New Systematics.* Edited by J. S. HUXLEY. Clarendon Press, Oxford.

INGRAM, V. M., 1957 Gene mutations in human haemoglobin: The chemical difference between normal and sickle cell haemoglobin. Nature **180**: 326.

JOHNSON, M. L., and M. J. WICKS, 1959 Serum protein electrophoresis in mammals—taxononic implications. Syst. Zool. **8**: 88–95.

KRISTJANSSON, F. K., 1963 Genetic control of two prealbumins in pigs. Genetics **48**: 1059–1063.

MCINDOE, W. M., 1962 Occurrence of two plasma albumins in the domestic fowl. Nature **195**: 353–354.

STORMONT, C., and Y. SUZUKI, 1963 Genetic control of albumin phenotypes in horses. Proc. Soc. Exptl. Biol. Med. **114**: 673–675.

(Reprinted from Nature, Vol. 182, pp. 65–66, July 5, 1958)

A Genetic Mechanism for 'Thread Protein' Polymorphism in Cattle

POLYMORPHISM in the 'thread proteins' of cattle sera has been described previously[1]. Four phenotypes have been found, depending on the presence or absence of one or both of a pair of threadlike zones, presumably proteins, resolved by starch-gel electrophoresis in phosphate buffer. The two zones are well separated, 'thread protein' T1 migrating more slowly than the $S\alpha$ zone, and T2 more rapidly. Individuals with only a T1 zone (phenotype T1) are readily and consistently distinguished from individuals with only a T2 zone (phenotype T2). Samples taken from the same animal at intervals up to 18 months showed the same phenotypes as the initial samples. However, individuals with both T1 and T2 zones (phenotype T1/T2) or neither zone (phenotype T0) are not differentiated consistently, T1/T2 sometimes appearing as T0. Phenotypes T1/T2 and T0, therefore, are best considered as one phenotype (T*) in practice.

The numbers of offspring obtained from the six possible matings between the three distinguishable phenotypes are shown in Table 1. The results in Table 1 were obtained (*a*) from a large self-contained local herd of pedigree Friesians, and (*b*) from matings picked at random from Friesian herds in various parts of England. Consideration of the mating results suggested that 'thread protein' polymorphism in cattle is controlled by three autosomal genes T^A, T^B and T^O, so that zone T1 is seen in the presence of T^A and zone T2 in the presence of T^B, while T^O does not evoke the appearance of either 'thread protein'. Phenotype T1 is thus an expression of the genotypes T^A/T^A or T^A/T^O, phenotype T2 of the genotype T^B/T^B or T^B/T^O, while phenotype T1/T2 has the genotype T^A/T^B and the true phenotype T^O the genotype T^O/T^O.

The gene frequencies T^A, T^B and T^O can be calculated from the relationships $p = (T^A)^2 + 2(T^A \times T^O)$, $q = (T^B)^2 + 2(T^B \times T^O)$ and $r = (T^O)^2 + 2(T^A \times T^B)$, where p, q and r are the observed frequencies of the phenotypes T1, T2 and T*. These equations are readily solved on an electronic computer, and I am indebted to J. C. Gower, of Rothamsted, for calculating gene frequencies. The equations are satisfied by two sets of frequencies; the correct set can be chosen by examination of the mating results.

Table 1. OBSERVED and EXPECTED MATING RESULTS IN BRITISH FRIESIANS

Mating (phenotypes)	No. of matings (observed/expected)	Phenotypes of offspring		
		T1 (obs./exp.)	T2 (obs./exp.)	T* (obs./exp.)
	A. 96 matings in self-contained herd			
T1 × T1	12/ 9·8	8/10·3	—	4/ 1·7
T1 × T2	14/20·4	4/ 3·3	5/ 3·3	5/ 7·4
T1 × T*	28/21·3	13/15·8	5/ 2·5	10/ 9·7
T2 × T2	5/10·7	—	4/ 4·3	1/ 0·7
T2 × T*	24/22·4	2/ 2·1	17/13·6	5/ 8·3
T* × T*	13/11·6	4/ 3·9	4/ 3·9	5/ 5·1
	B. 92 matings picked at random			
T1 × T1	7/ 5·7	6/ 5·9	—	1/ 1·1
T1 × T2	19/18·7	5/ 4·2	7/ 4·8	7/10·0
T1 × T*	18/15·7	6/10·0	2/ 1·6	10/ 6·4
T2 × T2	12/15·4	—	9/10·4	3/ 1·6
T2 × T*	30/25·8	4/ 2·6	19/17·3	7/10·3
T* × T*	6/10·8	1/ 1·8	3/ 1·8	2/ 2·4

T* = Combined phenotypes (T1/T2 + T0)
No. of matings: *A*, $\chi^2 = 7{\cdot}99$; $P > 0{\cdot}1$ (5 d.f.); *B*, $\chi^2 = 4{\cdot}25$; $P > 0{\cdot}5$ (5 d.f.).
Phenotypes of offspring: *A*, $\chi^2 = 10{\cdot}76$; $P > 0{\cdot}3$ (10 d.f.); *B*, $\chi^2 = 10{\cdot}41$; $P > 0{\cdot}3$ (10 d.f.).

From 213 animals in the local herd, p, q and r were found to be 0·320, 0·333 and 0·347 respectively, and the corresponding correct set of gene frequencies were T^A, 0·280, T^B, 0·290 and T^O, 0·430. From 184 cows, heifers and calves from the matings picked at random, p, q and r were 0·248, 0·408 and 0·343, giving T^A; 0·226; T^B, 0·338, and T^O, 0·437. Using these gene frequencies, the expected distribution of offspring in each set of data has been calculated and is shown in Table 1. Also the expected number of each of the six possible matings has been calculated in each case and compared with the actual number found. It will be seen that the observed and expected distribution of offspring agree when tested by the χ^2 test, as do the expected and observed numbers of matings. The results presented in Table 1 therefore support the genetic mechanism proposed.

Although 'thread proteins' have been found in dogs and pigs[3], no evidence of polymorphism has been found in these zones in these animals, so far. The significance of 'thread protein' polymorphism in cattle is not clear at this stage.

There does not appear to be any correlation between the thread protein phenotypes and the six β-globulin cattle phenotypes described previously[3].

I wish to thank the Directors of Lord Rayleigh's Farms Inc., and Strutt and Parker Farms, Ltd., for permission to bleed cattle, and the veterinary officers

of the Milk Marketing Board for collecting blood samples from A.I. (artificial insemination) herds.

G. C. ASHTON

The Animal Health Trust,
Livestock Research Station,
Stock, Essex.
March 24.

[1] Ashton, G. C., *Nature*, **180**, 917 (1957).
[2] Ashton, G. C., *Nature*, **179**, 824 (1957).
[3] Ashton, G. C., *Nature* (in the press).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Reprint from GENETICS, Vol. 51, No. 3, March, 1965. *Printed in U.S.A.*

SERUM AMYLASE (THREAD PROTEIN) POLYMORPHISM IN CATTLE

G. C. ASHTON[1]

Cattle Research Laboratory, C.S.I.R.O., Division of Animal Genetics, Rockhampton, Queensland, Australia

Received November 4, 1964

THREAD-PROTEIN polymorphism has been described in cattle (ASHTON 1958) and in pigs (ASHTON 1960). The thread-proteins (Figure 1) appear as very fine, somewhat wavy, threadlike zones on starch gel stained with protein stains. In agar electrophoresis in borate buffer at pH 8.6, the thread-proteins have the mobility of albumin, while in starch gel at pH 8.5 in phosphate buffer their mobility approximates that of the slow-alpha globulin.

The nature of the thread-proteins was not clear at the time of these publications. F. K. KRISTJANSSON (personal communication) working with pig serum noted that when stained gels were warmed for 5 to 10 minutes at 90° C the entire migration path behind the thread-proteins became depressed below the level of the remainder of the gel surface. This suggested some alteration in the

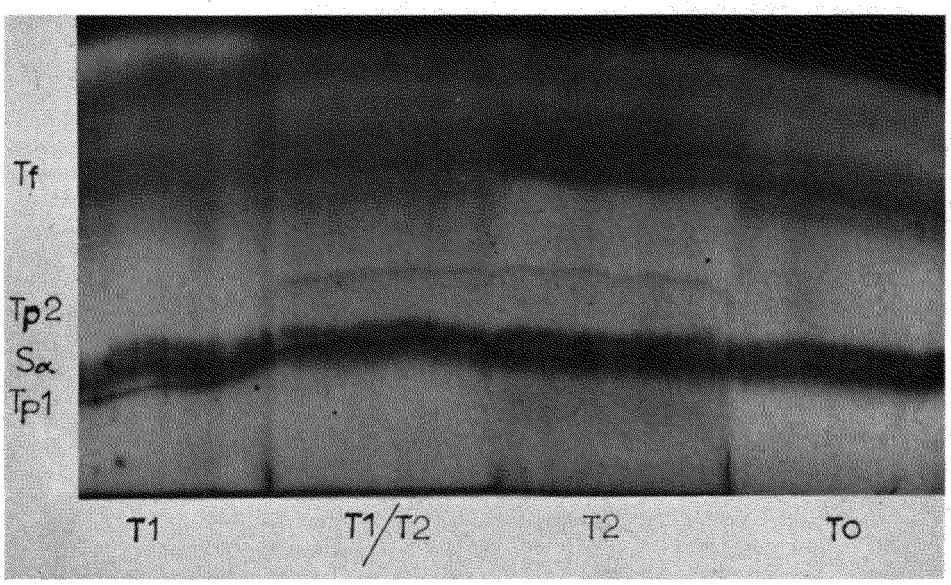


FIGURE 1.—Cattle thread-protein phenotypes as seen in phosphate buffer at pH 8.5 (ASHTON 1958). Tp 1, Tp 2, thread-proteins; Tf, transferrins (not well resolved in this buffer system); Sa, slow-alpha globulin.

[1] Present address: Department of Genetics, University of Hawaii, Honolulu.

gel structure due to the migration of the thread proteins. J. W. B. KING (private communication) suggested that the thread-proteins are in fact amylases, and has confirmed this hypothesis by suitable tests (to be published). The amylase activity of pig serum is much stronger than that of cattle serum, and the degradative action of the amylases of pig serum can be seen by incubating the unstained gel in a suitable buffer following electrophoresis.

Serum amylase polymorphism in cattle is rather difficult to see on unstained gels after incubation. However KING (private communication) has found that amylases from both pig and cattle serum can be seen after prolonged immersion in 0.1 percent p-phenylenediamine dihydrochloride in acetate buffer at pH 5.7, a stain used for detecting ceruloplasmin activity. This staining reaction, carried out at alkaline rather than acid pH, has been used to type the serum amylases of several hundred cattle to establish the mode of inheritance of this polymorphism.

MATERIALS AND METHODS

Starch gel electrophoresis: Best results were obtained with phosphate buffer and electrolyte although other systems also showed the polymorphism. The electrolyte contained 21 g of anhydrous disodium hydrogen phosphate per liter. Before making to volume this was adjusted to pH 7.6 with a saturated solution of potassium dihydrogen phosphate. The buffer used for preparing the gels was a 1/50 dilution of this electrolyte in water. The gels were prepared from hydrolysed starch (Connaught Laboratories, Toronto, Canada) by the procedure described by KRISTJANSSON (1963). Usually it was found necessary to increase the starch concentration above that recommended by the manufacturers to obtain sharp zones. For example a starch batch (204–1) recommended for use at 10.2 percent w/v was used at 12.0 percent w/v.

Electrophoresis was effected in a horizontal plane with a gel 3 mm thick. Serum samples were applied on pieces of No. 17 chromatography paper, removed 15 minutes after electrophoresis commenced. The voltage between the inner edges of the filter paper wicks on the surface of the gel was 9 v per cm of gel length, and the initial current was approximately 1.5 ma per cm of gel width. Final current was about 3 ma per cm width after 90 to 105 minutes. After this time the fast amylase zone had usually migrated about 2 cm.

After removal from the apparatus, the lower surface of the uncut gel was placed uppermost in a staining tray and the stain was added. The stain was prepared just before use by dissolving p-phenylenediamine dihydrochloride in a 1/25 dilution of pH 7.6 phosphate electrolyte at a concentration of 0.1 percent w/v. The gel was transferred to an incubator at 37°C. After about one hour the ceruloplasmin zones, stained blue, were clearly visible. Following incubation overnight these zones faded (Figure 2). The following morning the gels were transferred to a wash solution containing 225 ml methanol and 50 ml glacetic acetic acid per liter. The gels which were initially bluish-pink gradually lightened and the amylase zones appeared after about 15 to 30 minutes. Prolonged immersion in the wash solution caused the amylase zones to fade. It was found advisable to transfer the gels to water after about 90 to 120 minutes, the exact time being judged by the appearance of the amylase zones. Transfer to water intensified the zones.

The amylase zones appeared in reflected light as bluish-brown zones on a pink background after washing out. However phenotype identification was aided by viewing the gels in transmitted light, when the amylase zones appeared as transparent zones against the undigested translucent starch gel.

Samples: Serum samples from animals at the National Cattle Breeding Station, "Belmont," were examined. The animals sampled comprised the F_2 progeny of Brahman × Shorthorn, Brahman × Hereford, Africander × Shorthorn and Africander × Hereford cows, the cows themselves, and the sires of the F_2 progeny. A number of F_2 Shorthorn × Hereford animals were also examined.

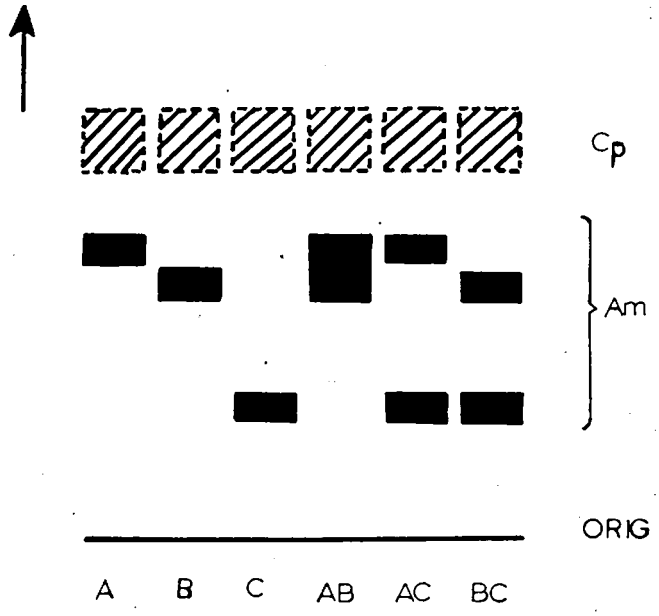


FIGURE 2.—Diagram showing the six cattle amylase phenotypes relative to the stained ceruloplasmin zones, which fade following overnight incubation. Cp, ceruloplasmin zones; Am, amylase zones. Arrow shows direction of migration towards anode.

Samples from cows in eight milking herds from Northern New South Wales were examined. Three of these herds were wholly Jersey, three predominantly Jersey, one predominantly Guernsey, and one predominantly Australian Illawarra Shorthorn. The female progeny of some of these cows, sired by artificial insemination from Sindhi × Jersey or Sahiwal × Jersey crossbred bulls were examined, and also the bulls responsible for the progeny.

RESULTS

Phenotypes: Six phenotypes were observed in the Brahman crossbred and Africander crossbred cattle from "Belmont," but only three of these six were observed in the dairy cattle. Of the six phenotypes (Figures 2, 3) three, Am A, Am B, and Am C, consisted of single zones with differing mobilities and these subsequently proved to be homozygotes. Two of the remaining three phenotypes, Am AC and Am BC, each showed two zones corresponding to the zones produced by the homozygotes, and these proved to be heterozygotes. The sixth phenotype, Am AB, showed a single broad zone (Figure 1). In the dairy cattle only Am B, Am BC, and Am C were present.

Mating data: The appearance of the phenotypes suggested that amylase polymorphism is controlled by three codominant alleles Am^A, Am^B and Am^C, each allele producing a single zone in starch gel with the zones differing in mobility. On this hypothesis phenotypes Am A, Am B, and Am C would be the products of genotypes Am^A/Am^A, Am^B/Am^B, and Am^C/Am^C respectively, and phenotypes Am AB, Am AC, and Am BC the products of genotypes Am^A/Am^B, Am^A/Am^C,

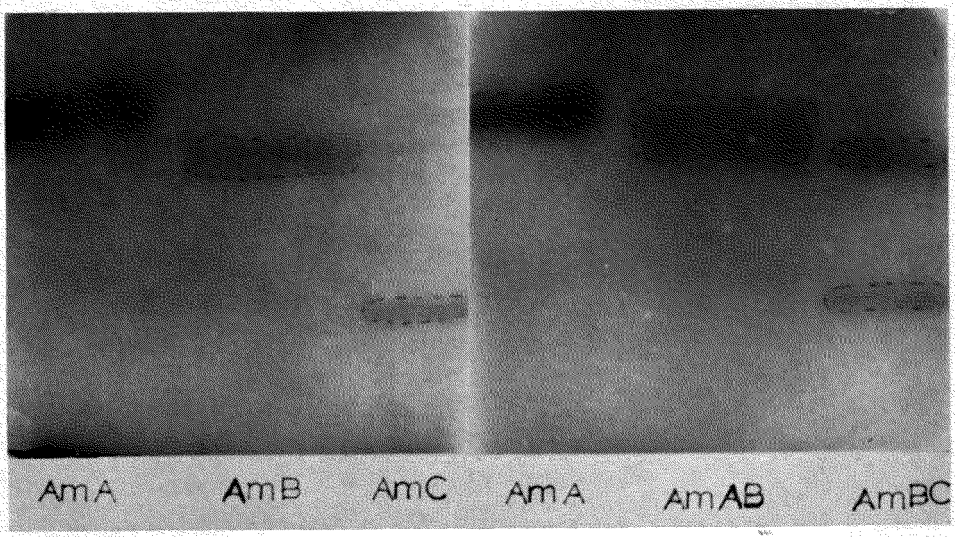


FIGURE 3.—Cattle serum amylase phenotypes after p-phenylenediamine staining. The zones were photographed by light transmitted through the gel and consequently appear darker than the background in the photograph instead of lighter. The amylase zones have been circumscribed in ink to improve contrast.

and Am^B/Am^C. Mating data obtained from the crossbred beef cattle (Table 1) and the dairy cattle (Table 2) confirmed this hypothesis. No exceptions to the hypothesis were encountered, and the observed and expected distribution of

TABLE 1

Amylase mating results from the "Belmont" Zebu-crossbred herd showing the observed distribution of phenotypes (numerator) and the distribution expected from the proposed three-allele hypothesis (denominator)

Phenotypes mated	Phenotypes of progeny					
	A	AB	AC	B	BC	C
A × AC	1/1	.....	1/1	.....	.....	.....
A × BC	.....	1/0.5	0/0.5	.....	.....	.....
A × C	.....	.....	2/2	.....	.....	.....
AB × AC	5/4.5	5/4.5	3/4.5	.....	5/4.5	.....
AB × BC	.....	8/3.75	0/3.75	3/3.75	4/3.75	.....
AB × C	.....	.....	6/5.5	.....	5/5.5	.....
AC × AC	6/4.75	.....	9/9.5	.....	.....	4/4.75
AC × BC	.....	6/5.75	5/5.75	.....	7/5.75	5/5.75
AC × B	.....	.....	0/0.5	.....	1/0.5	.....
AC × C	.....	.....	15/11.5	.....	.....	9/11.5
B × C	.....	.....	.....	.....	3/3	.....
BC × B	.....	.....	.....	2/2	2/2	.....
BC × BC	.....	.....	.....	0/2.5	8/5	2/2.5
BC × C	.....	.....	.....	.....	18/14/5	11/14.5
C × C	.....	.....	.....	.....	.....	6/6

TABLE 2

Amylase mating results from dairy cattle herds showing the observed distribution of phenotypes (numerator) and the numbers expected from the hypothesis proposed (denominator)

Phenotypes mated	Phenotypes of progeny		
	B	BC	C
B × B	18/18	.....	.....
B × BC	37/33.5	30/33.5	.....
B × C	.....	6/6	.....
BC × BC	13/10.5	20/21	9/10.5
BC × C	.....	3/3.5	4/3.5

TABLE 3

Distribution of amylase phenotypes in eight dairy herds, the derived gene frequencies, and the expected phenotype distribution calculated from these frequencies

Herd	Breed		Total	Phenotypes			Gene frequencies	
				B	BC	C	Am^B	Am^C
B	Jersey	Observed	60	27	27	6	0.675	0.325
		Expected		27.3	26.3	6.4		
G	Jersey	Observed	120	40	63	17	0.596	0.404
		Expected		42.6	57.8	19.6		
Sg	Jersey	Observed	75	37	31	7	0.700	0.300
		Expected		36.7	32.0	6.3		
W	Mainly Jersey	Observed	76	26	39	11	0.599	0.401
		Expected		27.3	36.5	12.2		
D	Mainly Jersey	Observed	173	42	98	33	0.526	0.474
		Expected		47.9	86.2	38.9		
O	Mainly Jersey	Observed	142	60	57	25	0.623	0.377
		Expected		55.1	66.7	20.2		
	Total of Jerseys	Observed	646	232	315	99	0.603	0.397
		Expected		234.9	309.3	101.8		
Sc	Guernsey	Observed	46	34	9	3	0.837	0.163
		Expected		32.2	12.6	1.2		
T	Illawarra Shorthorn	Observed	72	26	39	11	0.597	0.403
		Expected		27.3	36.5	12.2		

progeny did not differ significantly in any mating group.

Breed distribution of amylase types: Table 3 shows the distribution of cow genotypes for the eight dairy herds, and the derived gene frequencies for each herd. The observed and expected distribution do not differ significantly.

The gene frequencies for the crossbred beef cattle are strongly influenced by the experimental mating program in use at "Belmont," and for this reason are not considered in detail in this report. However it seems likely that Am^A is a feature of Brahman and Africander cattle and may be absent from the common

British breeds of cattle. It was not present in the Hereford × Shorthorn crosses and their descendants in the "Belmont" herd, nor was it found in the dairy cattle examined.

DISCUSSION

J. W. B. KING (personal communication) has suggested the locus symbol *Am* in place of *T* for thread-protein amylase activity in pigs. This more descriptive symbol has been adopted in the present work with cattle.

Four thread-protein phenotypes were recognized in British breeds of cattle by ASHTON (1958), T1 with a single slow-moving zone, T2 with a single fast-moving zone, T1/T2 with both zones, and T0 with no discernible zones (Figure 1). Comparison of thread-protein patterns after nigrosine staining, and amylase patterns after p-phenylenediamine staining, showed that thread-protein phenotype T2 is equivalent to amylase phenotype Am B, T1 to Am C, and T1/T2 to Am BC. Thread-protein phenotypes classed as T0 (no discernible zones) were found to show zones by the more sensitive phenylenediamine procedure. In most cases T0 phenotypes were found to be Am BC phenotypes, but in some cases they proved to be Am B or Am C.

It is apparent that the genetic hypothesis proposed for thread-protein polymorphism in cattle, involving a "silent" allele T^0 to account for the phenotypes not having detectable thread-protein zones, is incorrect. Phenotypes lacking amylase zones were not seen in the present study, although sometimes very faint zones detectable with certainty only in transmitted light were obtained.

Apart from cattle and pigs, thread-proteins, presumably amylases, have been seen in sheep, goats, dogs and horses, but they have not been found to be polymorphic in these species. BERK, KAWAGUCHI, ZEINEH, UJIHIRA and SEARCY (1963) have produced chromatographic evidence that at least three molecular forms of amylase occur in normal rabbit serum.

Serum amylase has not been detected in human serum by the method described, although very strong zones are obtained with human saliva. Results obtained by WILDING (1963) show that amylase exists unbound in human serum and the possibility of serum or salivary amylase polymorphism in humans should be considered.

I am indebted to DR. J. W. KING for information about the nature of thread-proteins, and for providing details of methods of detection. Thanks are due to MR. J. F. KENNEDY for access to cattle at the National Cattle Breeding Station, "Belmont," Rockhampton, Queensland, and to MR. R. W. HEWETSON of C.S.I.R.O. Division of Animal Genetics, Wollongbar, New South Wales for supplying blood samples from dairy cattle.

SUMMARY

The results of 178 matings in a beef cattle herd and 140 matings in eight dairy herds show that amylase polymorphism in cattle is controlled by three autosomal codominant alleles, Am^A, Am^B, and Am^C. Amylase polymorphism is equated with previously described thread-protein polymorphism. Am^A was present in the progeny from Brahman × British and Africander × British crossbreds, but not in the dairy cattle examined. Am^B and Am^C were present in all breeds examined.

LITERATURE CITED

ASHTON, G. C., 1958 A genetic mechanism for thread-protein polymorphism in cattle. Nature **182**: 65–66. —— 1960 Thread protein and β-globulin polymorphism in the serum proteins of pigs. Nature **186**: 99–992.

BERK, J. E., M. KAWAGUCHI, R. ZEINEH, I. UJIHIRA, and R. SEARCY, 1963 Chromatographic heterogeneity of rabbit serum amylase. Science **141**: 1182–1183.

KRISTJANSSON, F. K., 1963 Genetic control of two pre-albumins in pigs. Genetics **48**: 1059–1063.

WILDING, P., 1963 Use of gel filtration in the study of human amylase. Clin. Chim. Acta **8**: 918–924.

Aust. J. Exp. Biol. Med.
(in press).

(18)

STABILITY OF TICK RESISTANCE IN CATTLE:

ITS CORRELATION WITH VARIOUS GENETIC CHARACTERISTICS[1]

by J. FRANCIS and G. C. ASHTON

(From the Veterinary School, University of Queensland, Brisbane, Australia, and Department of Genetics, University of Hawaii, Honolulu, Hawaii)

Summary.

1. In 1961, when 41% of the cattle studied were of the Bos taurus breeds, the mean individual tick count was 9.8 on these animals and 1.6 on the Droughtmasters. From 1962 onwards the number of Bos taurus cattle fell to about 10%. Mean tick counts in 1962 were higher than in 1961, presumably due to the build-up of larvae that had occurred in 1961, but then fell to 2.1 on the European and 0.18 on the Droughtmaster cattle in 1964.

2. The relative tick resistance, or susceptibility, of the 51 Droughtmaster cattle remained very stable (rank correlation 0.9) over a three year period, despite the very low tick counts.

3. There was no correlation between tick resistance and the distribution of alleles at the following loci: haemoglobin, albumin, transferrin, post-albumins, and J soluble antigen.

4. There was a significant association between the distribution of amylase genes in the Droughtmaster cattle and tick resistance, and a similar but not significant association in the Bos taurus cattle.

5. There was a significant difference in both the mean [Ke^+] and packed cell volume between the European and Droughtmaster cattle, but these characteristics were not correlated with tick resistance within the Droughtmaster cattle.

---

(1) Partly supported by funds from the Australian Cattle and Beef Research Committee.

6. Visual assessment of Droughtmaster cattle, based largely on the recognition of "Brahman characteristics", gave a significant correlation with tick resistance. It is suggested, however, that there is no basis on which to make a visual assessment of resistance in pure Bos indicus or pure Bos taurus breeds.

INTRODUCTION

Domestic cattle of the world can be divided broadly into the non-humped Bos taurus cattle originating in the temperate regions of Europe and the humped Bos indicus or zebu cattle which originated in India or an adjoining region, and are now spread over much of China and Africa with India as the focus. Bos indicus cattle, including the Brahmans developed in the southern U.S.A., have a greater heat tolerance than Bos taurus, and are more resistant to many stress factors including a wide variety of infectious diseases (Francis, 1965a, b). Bos indicus cattle and various hybrids have become relatively common in coastal Queensland during the past 20 years and have attracted increasing interest because of their tick resistance and other characteristics of advantage in the tropics. The Droughtmaster is the most common of the hybrids developed in Australia and was produced by crossing Brahman bulls with cows of the British beef breeds. Droughtmasters, which are still in the process of being established, are red in colour and carry approximately half Brahman and half British " blood".

The numbers of ticks on Droughtmaster and Bos taurus cows grazing under the same conditions were reported by Francis and Little (1964). Bos taurus cattle carried 10.5 times as many engorged female ticks (Boophilus microplus, Canestrini) as Droughtmaster cattle and the level of infestation with ticks at any time was apparently related to the rainfall three months previously.

There is increasing interest in the possibility that one or more of the genetically controlled blood and serum factors in cattle may be associated with differential resistance to disease, differential fertility or differential production of economically useful products. This optimism is based on two premises. Firstly, differential resistance to disease and differential fertility are potent mechanisms for producing the differences in fitness necessary for maintaining a polymorphism balanced by heterosis. Secondly, it is becoming evident that major genes influence more than one character, and instances of pleiotrophy are accumulating.

Some progress has been made. Associations between transferrin type and fertility have been demonstrated in cattle (Ashton, 1965) and in pigs (Kristjansson, 1964). Pleiotrophic associations between milk yield and transferrin type have been found in cattle (Ashton, Fallon and Sutherland, 1964), and between butterfat percentage and specific blood group B alleles in cattle (Corneally and Stone, 1965). So far associations between disease susceptibility and blood factors have not been demonstrated in cattle, although the association between haemoglobin S and susceptibility to falciparum malaria in man (Alison, 1956), and between ABO blood groups and various afflictions of the gastro-intestinal tract in man (Clarke, 1962) are well-known and now considered authentic.

It is the purpose of the present paper to describe the stability of the relative tick counts over a period of three years and to report the results of attempts to relate various biochemical and other characteristics to tick resistance.

MATERIALS AND METHODS

The observations were made on a farm of 550 acres on the outskirts of Brisbane, latitude 27°S. All observations were made on adult cattle. The Droughtmasters were those in the breeding herd, and the Bos taurus cattle were Herefords and Friesians. In 1961 there were 60 Droughtmaster and 40 Bos taurus cattle on the farm which were allowed to graze as one herd. It will be seen from Table 1 that the number of Bos taurus cattle was reduced to 16 by 1962 whereas the Droughtmasters had increased to 143. There were smaller changes in subsequent years.

Ticks were counted on all cattle about 40 times a year, using the techniques described by Francis and Little (1964). Despite the low average counts, the Bos taurus cattle required four or five treatments with acaricide during the periods of high infestation in February and March. The fifty-one Droughtmaster cattle were given a subjective score, from 1 - 5, for tick resistance by four observers. This was based largely on the amount of "Brahman characteristics" (Francis, 1965) shown by each animal.

This herd was one of the three herds of Droughtmasters examined by Ashton, Francis and Ritson (1966) for transferrin, post-albumin, albumin, amylase and haemoglobin types. The protein genotypes were determined by starch-gel electrophoresis. The J-blood group phenotypes were determined by Dr. K. Bell. The erythrocyte potassium concentrations [Ke^+] were measured using the method of Evans (1963).

RESULTS

Stability of Tick Counts

In 1961 the average tick count on the Droughtmasters was 1.6 and on the Bos taurus cattle 9.8 ticks. From 1962 onwards there was a steady decline in tick numbers (Table 1) and by 1964 the mean count on the eleven most tick-

resistant Droughtmaster cattle was only 0.05, that is an average of one tick on every 20 animals.

The total tick counts for 1962 were used to place cattle in rank order of counts or susceptibility to infestation. Table 2 shows that in the 51 Droughtmasters the rank correlation between years was 0.9, and although the rank correlation was lower with the smaller number of European cattle, the least squares correlation was still significant.

Correlation of Characteristics with Tick Resistance

The characters examined fall into two groups, namely [Ke^+] and packed cell volume (p.c.v.) for which the mechanism of inheritance is unknown, and erythrocytic and serum factors for which the inheritance is reasonably well understood. In the first case it was possible only to assess the correlation between the quantity measured and mean tick count within each breed group. Table 1 shows the mean red cell [Ke^+] and p.c.v. for the 51 Droughtmasters arranged in five rank order groups. Neither character shows any obvious correlation with tick count, and this is confirmed by analysis of variance (Table 3) for both breeds.

Assessment of an association between disease susceptibility and phenotype may be made in several ways. The most sensitive method is to compare the frequency of infected and non-infected, or infested and non-infested, individuals between genotypes. Tick counts are not stable, however, varying with season and other factors and an all-or-none criterion of infestation cannot be applied. The most satisfactory alternative approach is to compare gene frequencies in rank order groups or groups of contrasting degrees of infestation.

Table 4 shows the gene frequencies for haemoglobin, albumin, postalbumin, transferrin, amylase and J-substance polymorphisms for five groups of Droughtmaster cattle. The five groups were composed of successive groups of ten animals arranged in decreasing rank order based on the 1962 counts. The

least infested group had eleven animals. J-substance determinations were done on all animals. However, one animal in the most infested group was not available for blood sampling for protein polymorphisms, and the data for this group (except for J-substance) refer to nine animals.

There is no evidence of a consistent trend in gene frequencies in the haemoglobin, albumin, post-albumin, transferrin or J-substance systems. There is a trend, however, in the frequencies of the $\underline{Am^B}$ and $\underline{Am^C}$ alleles. $\underline{Am^B}$ increases in frequency as the mean tick burden for the group decreases, while $\underline{Am^C}$ frequency decreases. Expressed as a simple correlation, this is significant ($r = -0.923$, 3 d.f., $P < 0.05$). This prompted a closer investigation of serum amylase — tick burden relationship. Table 4 shows the mean log counts for each genotype and their standard errors. Analysis of variance showed that the variance between genotypes was not significantly greater than that within genotypes. However comparison of the number of amylase A, B, and C alleles in the two most heavily infested groups of animals with that in the two least infested groups of animals showed a significant difference ($\chi^2 = 6.414$, 2 d.f., $P < 0.05$). This difference seems to be due mainly to a lower frequency of $\underline{Am^B}$ in the least infested groups.

Table 4 also shows gene frequencies for twelve Bos taurus cattle, arranged in two groups, viz., the six most and the six least infested animals. Although two groups are not informative with respect to trends it is noteworthy that the frequency of $\underline{Am^B}$ is greater in the lesser infested group. The distribution of alleles (Table 4) does not differ significantly between the two groups of Bos taurus cattle, although the trend is in the same direction as that seen in the Droughtmaster cattle.

Subjective Score of Tick Resistance

Four observers scored the Droughtmaster cattle for tick resistance and the mean scores of the groups are shown in Table 1. There was a regular gradation of score except that the most resistant cattle had a lower score than the previous group. However, the analysis of variance in Table 6 shows that there was a significant difference ($P < 0.015$) between groups. When these cattle were arranged in two equal groups of high and low tick resistance there was a significant difference ($P < 0.01$) between the scores of the two groups.

DISCUSSION

Tick Counts

The mean tick counts in 1962 were higher than in 1961 presumably due to the build up of larvae which had occurred in that year but they fell consistently from 1961 to 1964. Rainfall which could have influenced the results was very similar in the years 1961 - 1964. Concerning the overall tick counts it should be said that the district is one in which *Bos taurus* cattle become heavily infested unless control measures are taken. The very low level of tick infestation in the Droughtmaster cattle was presumably due to the fact that the great majority of female larval ticks attaching to Droughtmaster cattle die before reaching maturity (Francis and Little, 1964). There are thus progressively fewer mature female ticks to produce eggs, and the number of larvae on the pasture steadily declines. The tick counts on Droughtmaster cattle would probably have been even less if the tick larvae had not been matured to some extent by the *Bos taurus* cattle. Conversely, if all the cattle on the property had been *Bos taurus*, the tick infestation would probably have been far greater unless the cattle were frequently treated with acaricide. The ability of Brahman-type cattle to "cleanse" pastures of tick infestation was noted by American observers at the turn of the century after these cattle were introduced

into Texas. Although the cleansing is never absolute it is obviously of the greatest practical importance in enzootic areas. As indicated the observations on Droughtmaster cattle in this study show that cattle with about half Brahman "blood" very rarely need dipping in Southern Queensland. To achieve this result in Northern Queensland cattle with 3/4 Brahman blood are probably necessary.

These overall facts were reported by Kelley (1943) who pointed out that tick resistance was closely related to the amount of Brahman "blood". This clearly indicates that tick resistance is strongly inherited, and the fact that relative tick counts are so stable from year to year shows that tick resistance is a generally stable genetic characteristic of individual cattle although it can, of course, be influenced by nutrition or other factors and tick counts can be increased by heavy exposure to infestation.

This would appear to be the first detailed report on the stability of relative tick counts, and it is of particular interest that the stability has been maintained despite the very low average counts.

Correlation of Characteristics with Tick Resistance

It was hoped that one or other of the characters studied would be related to observed tick resistance and therefore could be used as a "gene marker" in selecting cattle for tick resistance. As anticipated most of the characters studied (albumin, post-albumin, transferrin, amylase, [Ke^+], and p.c.v.) showed significant breed differences in gene frequencies in line with those reported in the literature (Table 3). Only one character, namely amylase, showed any correlation with tick resistance within a breed. The data for both Droughtmaster and Bos taurus cattle suggest that the frequency of Am^B is higher in the least infested groups of animals. Clearly, many factors will influence tick

resistance, and the mean level of genetic resistance in a breed will be the sum of all these characters. Analysis of variance showed that there were no significant differences between the mean values for tick burden in each of the amylase genotypes. However, the mean total tick count for all Droughtmasters carrying Am^B was 86 counts, and for those not carrying this allele was 233 counts. Similarly in the Bos taurus cattle the mean total tick count for animals carrying the B allele was 847 counts, and for the one animal not carrying this allele was 2296 counts. Selection of Am^B carrying animals in both these herds would have been expected to produce an overall decrease in tick burden. It remains to be seen whether similar results will be obtained with other herds and breeds of cattle. It is found quite commonly that associations between a genetic character and disease attack rate or susceptibility are not highly repeatable between workers. A considerable number of observations by independent laboratories are necessary before observations of the sort reported here showing a relationship between amylase type and tick infestations may be considered valid.

It is interesting that visual appraisal of Droughtmaster cattle did give a significant correlation with tick resistance although this was less precise than had been hoped. There was significant variation in scoring between operators (Table 6), and appraisal will probably be improved when this source of variation is reduced. Kelly (1943) concluded, on the basis of extensive observations, that tick resistance was fairly closely related to the amount of Brahman blood in hybrid cattle and there is a widely held view that this can be approximately assessed by visual appraisal.

It has been realized since 1915 (Francis, 1965b) that some Bos taurus cattle are considerably more tick resistant than the majority, but it is unlikely that these resistant cattle could be recognized visually as Little and Francis

(1964) found that tick resistance was not necessarily related to coat length. Again, a few zebu or Brahman cattle are considerably more susceptible to ticks than the average but there is no visual method of recognizing these animals. Unless more extensive observations show that the amylase locus is correlated with tick resistance in Brahman hybrid cattle, visual scoring provides the best estimate of tick resistance in these animals. Although the narrow range of variability of subjective scores indicates that selection of individual cattle by this method would be unreliable, groups of cattle selected by this method should be more tick resistant.

ACKNOWLEDGMENTS

We are indebted to Mr. M. Smith, Mr. G. Parker and Mr. M. N. Dennis for technical assistance, and to Mr. R. Sheard and Dr. K. Bell for assistance in the preparation of the paper.

REFERENCES

Allison, A. C., (1956): "Population Genetics of Abnormal Human Haemoglobins". Acta Genet. Statist. Med., 6: 430.

Ashton, G. C., (1965): "Cattle Serum Transferrins: A Balanced Polymorphism?" Genetics, 52: 983.

Ashton, G. C., Fallon, G. R. and Sutherland, D. N., (1964): "Transferrin (β-Globulin) Type and Milk and Butterfat Production in Dairy Cows." J. Agric. Sci., 62: 27.

Ashton, G. C., Francis, J., and Ritson, J. B., (1966): "Distribution of Transferrin, Albumin, Post-Albumin, Amylase and Haemoglobin Genotypes in Droughtmaster Cattle." Aust. J. Agric. Res., submitted.

Clarke, C. A., (1962). "Genetics for the Clinician". Blackwell Scientific Publications, Oxford.

Conneally, P. M. and Stone, W. H., (1965): "Association Between a Blood Group and Butterfat Production in Dairy Cattle". Nature, 206: 115.

Francis, J., (1965a): "Definition and Use of Zebu, Brahman or Bos Indicus Cattle." Nature, 207: 13.

Francis, J., (1965b): "Resistance of Zebu and Other Cattle to Tick Infestation and Babesiosis with Special Reference to Australia." Brit. Vet. J. (Accepted for publication).

Francis, J. and Little, D. A., (1964): "Resistance of Droughtmaster Cattle to Tick Infestation and Babesiosis". Aust. Vet. J., 40: 247.

Kelley, R. B., (1943): "Zebu-cross Cattle in Northern Australia - an Ecological Experiment." C.S.I.R.O. Bull., No. 172.

Kristjansson, F. K., (1964): "Transferrin Types and Reproductive Performance in the Pig." J. Reprod. Fertil., 8: 311.

TABLE 2

Statistical Analysis of the Relationship of Tick Numbers in 1962, 1963 and 1964

Cattle	Years compared	Rank correlation	Least squares correlation
51 Droughtmaster	1962/63	0.93	0.67 ($P < 0.001$)
	1962/64	0.90	0.65 ($P < 0.001$)
12 *Bos taurus*	1962/63	0.73	0.73 ($P < 0.01$)
	1962/64	0.44	0.67 ($P < 0.05$)

TABLE 1

Cattle Population and Tick Numbers, [Ke^+], P.C.V. and Subjective Grading on 51 Droughtmaster and 12 Bos taurus Cattle

Cattle Population in June	Bos taurus / Droughtmaster	1962	1963	1964	Mean tick counts 1962/64	Mean Red Cell [Ke^+]	P.C.V.	Mean subjective scores of tick resistance
		16	15	12				
		143	163	179				
		Mean Individual Tick Count*						
Numbers and type of cattle in various groups	6 Bos taurus	22.30	4.75	2.25	9.67	19.9	32.4	
	6 " "	9.56	2.67	1.94	4.72	21.9	29.8	
	10 Droughtmaster	7.99	1.81	.32	3.37	14.2	36.9	2.85
	10 "	3.10	.76	.24	1.37	16.3	37.4	2.95
	10 "	1.68	.36	.19	.74	13.3	38.5	3.25
	10 "	.84	.39	.10	.44	16.2	39.5	3.63
	11 "	.21	.43**	.05	.23	13.9	36.5	3.20
Mean	12 Bos taurus	15.94	3.71	2.10	7.22	20.9	30.8	
Mean	51 Droughtmaster	2.62	0.68	0.18	1.16	14.8	37.7	

* Figures are mean counts of ticks on individual animals on any one occasion.

** This discrepant figure is due to high counts on cow 46 during 1963. If they are excluded the mean figure is 0.14.

TABLE 3

Analysis of Variance of [Ke^+] and Packed Cell Volume (P.C.V.) for the Tick Counts in the Rank Order Groups Shown in Table 1

Comparison	D.F.	[Ke^+]			P.C.V.		
		MSS	F	P	MSS	F	P
Within Droughtmasters							
Between tick groups	4	19.6	0.5	N.S.	14.5	1.1	N.S.
Within tick groups	46	38.1			13.6		
Within European							
Between tick groups	1	12.6	0.4	N.S.	20.0	1.3	N.S.
Within tick groups	10	35.3			15.6		
Between breeds							
Between breeds	1	364.2	10.1	<0.01	397.2	28.2	<0.001
Within breeds	61	36.0			14.1		

TABLE 4

Gene Frequencies in Groups of Cattle Graded for Tick Resistance

Locus	Allele	Droughtmasters Mean tick numbers 7.99	3.10	1.68	0.84	0.21	Bos taurus Mean tick numbers 22.30	9.56
Amylase	Am^A	0.333	0.400	0.300	0.250	0.273	0.083	0.083
	Am^B	0.111	0.150	0.300	0.350	0.409	0.584	0.667
	Am^C	0.556	0.150	0.400	0.400	0.318	0.333	0.250
Albumin	Alb^A	0.563	0.550	0.400	0.556	0.409	0.917	0.917
	Alb^B	0.437	0.450	0.600	0.444	0.591	0.083	0.083
Haemoglobin	Hb^A	0.786	0.778	0.722	0.667	0.950	0.833	0.917
	Hb^B	0.214	0.222	0.278	0.333	0.050	0.167	0.083
Post-albumins	Poa^F	0.374	0.550	0.450	0.444	0.682	0.417	0.083
	Poa^S	0.556	0.450	0.550	0.556	0.318	0.583	0.917
Transferrin	Tf^A	0.125	0.450	0.100	0.222	0.182	0.333	0.167
	Tf^B	--	0.050	--	0.056	0.091	--	--
	Tf^{D1}	0.125	0.050	0.050	0.000	0.091	0.083	0.250
	Tf^{D2}	0.438	0.250	0.450	0.389	0.318	0.584	0.583
	Tf^F	0.125	0.050	0.050	0.056	0.091	--	--
	Tf^E	0.187	0.150	0.350	0.279	0.227	--	--
J*	J^+	0.850	0.750	0.650	0.850	0.950	0.707	0.816
	J^-	0.150	0.250	0.350	0.150	0.050	0.293	0.184

* Considered as two alleles

$J^+ = J^+/J^+$ & J^+/J^-, & $J^- = J^-/J^-$

TABLE 5

Distribution of Amylase Alleles in High and Low Infested Groups

Breed	Group	Total No. of Cells			Gene Frequencies		
		Am^A	Am^B	Am^C	Am^A	Am^B	Am^C
Droughtmaster	High(1)	14	5	19	0.368	0.132	0.500
	Low(2)	11	16	15	0.262	0.381	0.357
		$\chi^2 = 6.41$, 2 d.f., $P < 0.05$					
Bos taurus	High(3)	1	7	4	0.083	0.584	0.333
	Low(4)	1	8	3	0.083	0.667	0.250
		$\chi^2 = 0.07$, N.S.					

(1) 19 animals from the two most infested groups shown in Table 3, with mean tick counts of 7.99 & 3.10.

(2) 21 animals from the two least infested groups shown in Table 3, with mean tick counts of 0.84 & 0.21.

(3) 6 animals comprising the most infested group shown in Table 3, with mean tick count of 22.30.

(4) 6 animals comprising the least infested group shown in Table 3, with mean tick count of 9.56.

TABLE 6

Subjective Scores of Droughtmaster Cattle

Comparison	DF	Mean S.Sqs.	F
Between Operator	3	3.33	2.80 ($P < 0.05$)
Between Groups	4	3.90	3.28 ($P < 0.01$)
Error	192	1.19	

Aust. J Biol. Sci.
(in press)

(19)

DISTRIBUTION OF TRANSFERRIN, ALBUMIN, POST-ALBUMIN, AMYLASE AND HEMOGLOBIN GENOTYPES IN DROUGHTMASTER CATTLE

C. C. Ashton, J. Francis & J. B. Ritson

Heterozygote superiority is the most likely way in which a polymorphism may be balanced (Fisher, 1930) although there are other possible mechanisms (Haldane & Jayakar, 1963). Direct demonstration of heterozygote superiority and its consequent effect on polymorphic balance has been made in certain invertebrate species for some morphological characters and for chromosomal inversions (Ford, 1964). In vertebrate confirmation of Fisher's mathematical demonstration has been made only in the case of sickle cell hemoglobin in man (Allison, 1953) and serum transferrins in cattle (Ashton, 1965). As it is likely that the nature of the heterozygote advantage will be expressed through differential genotype survival in utero, differential survival to reproductive age and differential fertility, accurate estimates of these parameters are necessary. Measurement of some of these parameters is difficult in man, but in cattle most of the necessary data may be collected fairly readily. The widespread use of artificial insemination for dairy cattle permits measurement of fertility differences and differential survival in utero, while beef cattle populations permit measurement of segregation ratios and differential survival to reproductive age. Collection of data of these sort for serum transferrins in cattle has resulted in the elucidation of a possible balance mechanism for the polymorphism, and the presentation of a model which may be used as a basis for more stringent tests (Ashton, 1965).

If a polymorphism is maintained by heterozygote advantage it is likely (but not necessary) that a random population will show an excess of heterozygotes.

Individual herds of cattle are seldom random populations. However, herds which approach the ideal most closely are foundation herds in which the animals have been drawn from a variety of sources in an effort to get a representative stock. The recently established C.S.I.R.O. Droughtmaster herd at Townsville, Queensland, and the University of Queensland Droughtmaster herd at St. Lucia, Queensland, are both foundation herds of this type. These herds are of particular interest because they are composed of Droughtmaster cattle, which is a relatively recently established breed produced by selection from mainly Brahman x Shorthorn crossbred cattle. Several genes of interest, e.g., $\underline{Tf}^{B}$, $\underline{Tf}^{F}$, $\underline{Alb}^{B}$, $\underline{Am}^{C}$, and $\underline{Hb}^{B}$ not present in theparental European beef breeds used in founding the Droughtmaster breed have been introduced from the parental zebu stock. This provides opportunities for detecting heterozygote advantage not present in European breeds.

The object of the surveys reported here was to determine if cattle protein polymorphisms other than serum transferrin exhibit heterozygote advantage, as evidenced by the presence of an excess of heterozygotes in the populations studied.

MATERIALS & METHODS

Droughtmaster cattle. Details of the history and genetic compositions of the Droughtmaster breed have been presented by .

Herds sampled. Two herds of Droughtmaster cattle were bled. The first herd (C.S.I.R.O. Division of Tropical Pastures, Lansdowne Park, Townsville, Queensland) was founded by assembling cows from seventeen different herds of Droughtmaster herd. This herd has two populations of breeding cows, one comprising 93 classified breeds, i.e., meeting all requirements of the Droughtmaster herd book, and the other comprising 145 Droughtmaster or Droughtmaster-type cows, referred to subsequently as unclassified breeders.

The second herd belonging to the Veterinary School, University of Queensland, St. Lucia, Queensland, was established in 196 with the object of producing a herd of superior Droughtmaster cattle selected on the basis of type, tick resistance, and other factors of economic importance to the beef cattle industry in Queensland. At the time of sampling it comprised animals of which 123 were sampled.

Methods. The protein polymorphisms were typed by starch-gel electrophoresis. Transferrins and post-albumins were typed on the same gel (Ashton, 1964a, 1965c). Albumins (Ashton, 1964b) and amylases (Ashton, 1965b) were each determined on separate gels. Haemoglobin genotypes were determined on red cell haemolysates with the system used for transferrin typing.

RESULTS

Transferrin, post-albumin and amylase genotypes were determined on 93 cows in the Townsville classified breeders population, on 145 unclassified breeders at Townsville, and on 123 cows at St. Lucia. The albumin type of one classified breeder was not determined nor were the haemoglobin types of two classified and two unclassified breeders. The distribution of determined genotypes is shown in Table 1.

Gene frequencies were derived from the genotype distributions by gene counting (Smith, 195) and are shown in Table 2. Heterogeneity between the three populations for a given system was assessed by χ^2 analysis (Snedecor, 1956) by comparing the observed number of genes of each type for each population with that expected from the pooled values for the three populations. The distribution of transferrin genes did not differ significantly between the three populations

(χ^2 = 12.3, 10 d.f., P > 0.2), nor did the distribution of post-albumin genes (χ^2 = 0.26, 2 d.f., P > 0.8) or the distribution of hemoglobin genes (χ^2 = 0.23, 2 d.f., P > 0.8). Pooled gene frequencies for these three systems are shown in Table 2.

The distribution of amylase genes showed significant heterogeneity between populations (χ^2 = 189.9, 4 d.f., P < 0.01). The amylase gene frequencies of the two Townsville populations were not significantly different, but both differed significantly (P < 0.01) from the St. Lucia herd. The distribution of albumin genes also displayed significant heterogeneity between populations (χ^2 = 16.1, 2 d.f., P < 0.01), and while the two Townsville populations did not differ significantly from each other, each was significantly different (P < 0.01) from the St. Lucia herd.

The distribution of homozygotes and heterozygotes for each population and each polymorphism is shown in Table 3. Heterogeneity between populations in the distribution of homozygotes and heterozygotes for each polymorphism was tested by χ^2 analysis, comparing the individual distributions with the pooled distributions. Only the amylase system showed significant heterogeneity between populations in the relative proportion of homozygotes and heterozygotes (χ^2 = 6.49, 2 d.f., P < 0.05). The observed distribution of homozygotes and heterozygotes was then compared with the distribution expected from the gene frequencies shown in Table 2. There was a consistent and significant (P < 0.05) excess of transferrin heterozygotes, and a consistent and significant (P < 0.01) excess of albumin heterozygotes. The distribution of post-albumin, hemoglobin and amylase homozygotes and heterozygotes did not differ from expectation.

DISCUSSION

Gene frequencies calculated within herds are not necessarily representative

of breed gene frequencies. The three populations studied however were produced by admixture of selected cows from several sources, and probably represent as good a random sample as can be obtained by more sophisticated sampling techniques. The consistency of the distribution of transferrin, post-albumin and hemoglobin genotypes between the three populations supports the contention that the gene frequencies calculated for these systems will probably be close to the breed frequencies. The two Townsville populations do not differ in the distributions of amylase or albumin genotypes, which suggests that the distinction between classified and unclassified breeders may be more apparent than real. However, the distributions of genotypes in both these systems differs significantly between Townsville and St. Lucia. It is rather unlikely that limited use of sires per se has contributed to this difference in view of the consistency of the transferrin, post-albumin and hemoglobin distributions. It is possible that differential selection in the two herds has produced these gene frequency differences, although there is no indication of the nature of the differential selection.

The Droughtmaster Breeders Association permits classification of cows with between 3/8 and 5/8 zebu genes. Assuming that the mean constitution is one half British (mainly beef Shorthorn, with some Hereford and some Devon) and one half zebu (mainly Brahman) it is possible to calculate gene frequencies for the hypothetical parental zebu stock. This requires a knowledge of the gene frequencies of the parental British stock. For the hemoglobin and albumin systems this is known accurately, because in each system one of the two alleles present in zebus is absent in British beef cattle. The transferrin gene frequencies in beef Shorthorns and other British beef breeds are known with less accuracy, although Tf^{B} and Tf^{F} are absent. The frequencies for Tf^{A}, "Tf^{D}", and Tf^{E}, and for Pos^{F}

and $\underline{Po^C}$ are taken from unpublished studies on Queensland cattle. Insufficient data for amylase distribution are available for computing frequencies in British breeds.

Table 4 shows the gene frequencies for the hypothetical parent zebu stock, assuming the present admixture of British and zebu genes in Droughtmaster cattle is 1:1. There are no published gene frequencies for Brahman cattle as such, except for the hemoglobin system. Crockett, Koger and Chapman (1963) show genotype distributions for 182 Brahman cattle in Florida. Gene frequencies calculated from their data gave $\underline{Hb^A} = 0.541$, $\underline{Hb^B} = 0.447$ and $\underline{Hb^C} = 0.012$. These values differ markedly from the values calculated for the hypothetical parental stock. The calculated values, however, agree much more closely with the frequencies given by Lehmann and Rollinson (1958) for zebu cattle in East Africa, i.e., $Hb^A = 0.678$, $\underline{Hb^B} = 0.322$.

The only published data for transferrin post-albumin and albumin gene frequencies of zebu cattle are for East African cattle (Ashton and Lampkin, 1965a,b). The transferrin and post-albumin gene frequencies of these cattle were similar to the values calculated for the parental zebu stock (Table 4), although the albumin frequencies do not correspond. It seems that the population from which were drawn the several groups of imported zebu bulls that were the ancestors of the present day Australian Droughtmaster may have been similar in genetic condition to the zebu cattle found in East Africa today.

Transferrin polymorphism in cattle is maintained by heterozygote superiority in utero (Ashton, 1965). In many populations of cattle which have been examined heterozygote superiority leads to an excess of heterozygotes in the population. However, some Danish investigations (Larsen et al.) have not revealed an overall excess of heterozygotes, although analysis of the data show the expected excess from certain matings, obscured by maternal-fetal incompatibility (Ashton,

1965). Failure to show an overall excess of heterozygotes may be due in part to typing errors when the frequency of Tf^{D1} and Tf^{D2} are not very different and Tf^{E} is infrequent. Under these conditions about half of the animals typed as TfDD will be $Tf^{D1}D^{2}$ heterozygotes.

The three populations of Droughtmaster cattle examined here each show an excess of heterozygotes and overall this is significant with no between-population heterogeneity. The extent of the excess, 7.6%, is similar to that reported previously (Ashton and Fallon, 1962; and Lampkin, 1965). Robertson (1965) has drawn attention to the possibility of reporting an artefactual excess of heterozygotes in population of farm livestock. The gene counting methods of estimating gene frequency (Smith, 1957) usually employed assume that the population is panmictic, which in the case of cattle is not so. Extension of these gene frequency estimates to the computation of expected numbers of homozygotes and heterozygotes will be biassed by the number of sires in use in the population. An excess of heterozygotes will be obtained from this type of calculation, the extent of the excess being $1/8S$, where S is the number of sires in use in the herds sampled. In herds based on a single sire the artefactual excess will be 12 1/2%, but with 12 sires the excess will reduce to about 1%. In the populations investigated here the actual number of sires producing the cows in the Townsville populations exceeded twelve. The significant excess of transferrin heterozygotes is not likely to be due to minimum use of sires. However, if this were the reason one would expect a similar excess in other systems, and neither the hemoglobins nor post-albumin show a significant excess. A previous study (Ashton and Lampkin, 1965) also failed to reveal a significant excess of post-albumins, although there was an excess of transferrin heterozygotes.

An excess of transferrin heterozygotes has been demonstrated before, and this observation and others lead to a model for a balanced polymorphism (Ashton, 1965). Demonstration of an excess of heterozygotes in a population is therefore an indication of a polymorphism maintained by heterozygote advantage. Thus, the observation of a consistent and highly significant excess of albumin heterozygotes in the three populations studied is important. This system has not been examined extensively, but preliminary segregation data (Ashton, 1965) from a different population showed a significant discrepancy from expectation in the mating Alb A ♂ x Alb AB ♀, an excess of heterozygotes being produced. The reciprocal mating Alb AB ♂ x Alb A♀ produced a non-significant excess of homozygotes. These results i.e., the excess of heterozygotes in random populations, the excess of heterozygotes from A ♂ x AB ♀ matings where incompatibility is not possible, and a deficiency of heterozygotes from AB ♂ x A ♀ matings in which maternal-fetal incompatibility is possible, are very similar to findings with cattle transferrins. If validated these effects will be sufficient to produce a balanced polymorphism in the cattle albumin system. Data on the relative fertility of cows and bulls homozygous and heterozygous for albumin type are being collected.

No excess of heterozygotes was found in the post-albumin hemoglobin or amylase systems.

SUMMARY

Three hundred and sixty one cows from two herds of Droughtmaster beef cattle, representing three cattle populations, were typed for five protein polymorphisms. The gene frequencies for the transferrin, post-albumin and hemoglobin polymorphisms did not differ between the three populations. The gene frequencies for the

albumin and amylase polymorphisms were consistent in two populations (classified and non-classified breeders) in one herd, but the frequencies differed significantly in the two herds.

There was a consistent excess of transferrin heterozygotes in the three populations, which was significant -- overall ($P < 0.05$) and averaged 7.6%. There was also a significant ($P < 0.01$) and consistent excess of albumin heterozygotes which averaged 22%. The hemoglobin, post-albumin and amylase systems did not show an excess of heterozygotes.

TABLE 1. DISTRIBUTION OF SERUM PROTEIN GENOTYPES IN THREE POPULATIONS OF BROUGHTON-A-GONE CONS.

System	Genotype	Population		
		Classified Breeders at Kennyville	Unclassified Breeders at Kennyville	St. Lucia herd
Transferrin	A/A	5	5	11
	A/B	8	6	5
	A/D^1	5	5	2
	A/D^2	22	26	25
	A/E	10	20	14
	A/F	5	14	3
	B/B	0	2	0
	B/D^1	0	1	0
	B/D^2	3	8	9
	B/E	2	3	5
	B/F	2	2	3
	D^1/D^1	0	0	0
	D^1/D^2	2	0	7
	D^1/E	1	4	0
	D^1/F	1	2	0
	D^2/D^2	5	10	11
	D^2/E	8	14	20
	D^2/F	6	10	5
	E/E	3	8	1
	F/E	4	5	2
	F/F	1	0	0
Post Albumin	F/F	26	40	37
	F/S	46	74	61
	S/S	21	31	25

Table 3. Continued

System	Genotype	Population		
		Classified Breeders at Townsville	Unclassified Breeders at Townsville	St. Lucia herd
Amylase	A/A	0	1	17
	A/B	3	9	15
	A/C	3	4	30
	B/B	31	64	8
	B/C	43	46	27
	C/C	13	21	26
Haemoglobin	A/A	72	111	92
	A/B	18	31	31
	B/B	1	1	0
Albumin	A/A	26	43	13
	A/B	55	83	77
	B/B	11	19	33

TABLE 2. GENE FREQUENCIES DERIVED FROM DISTRIBUTION OF GENOTYPES IN TABLE 1.

System	Alleles	Population			
		C.S.I.R.O.	Townsville	University of Queensland St. Lucia(1)	Pooled
		Classified breeders(1)	Unclassified breeders(1)		
Transferrin	A	0.323	0.276	0.209	0.292
	B	0.081	0.083	0.089	0.084
	D^1	0.048	0.041	0.037	0.042
	D^2	0.274	0.272	0.358	0.302
	E	0.167	0.214	0.175	0.188
	F	0.107	0.114	0.052	0.092
Post Albumin	F	0.527	0.531	0.549	0.536
	S	0.473	0.469	0.451	0.464
Amylase	A	0.032	0.032	0.321	(2)
	B	0.581	0.631	0.236	
	C	0.387	0.317	0.443	
Hemoglobin	A	0.890	0.885	0.874	0.882
	B	0.110	0.115	0.126	0.118
Albumin	A	0.582	0.583	0.419	(2)
	B	0.418	0.417	0.581	

(1) No. of individuals given in text.

(2) Pooled values not compiled because of significant heterogeneity between populations.

SECTION "C": SPECIES OTHER THAN CATTLE

Paper No.	Title	Describes: -
20	Zone electrophoresis of mammalian sera in starch gels. (1957)	-early work on starch gel electrophoresis.
21	β-globulin polymorphism in cattle, sheep, and goats. (1958)	-first description of serum polymorphism detected by paper electrophoresis.
22	Polymorphism in the β-globulins of sheep. (1958)	-first description of transferrins in sheep.
23	Further β-globulin phenotypes in sheep. (1958)	-further Tf types in sheep.
24	Serum transferrins in merino sheep. (1963)	-further Tf types in merino sheep.
25	Serum protein variations in horses. (1958)	-first report of serum protein variation in horses.
26	Thread protein and β-globulin polymorphism in the serum proteins of pigs. (1960)	-first description of amylases and transferrins in pigs.
27	Serum β-globulin polymorphism in mice. (1961)	-first description of a now widely-used buffer system for starch gel electrophoresis.
28	Serum transferrins in some African antelopes. (1965)	-transferrin variation in eland and various gazelles.
29	C5 types of serum cholinesterase in a Brazilian population. (1966)	-significant deviations of segregation, and other effects, at the human E_2 locus.

(Reprinted from Nature, Vol. 179, pp. 824-825, April 20, 1957)

Zone Electrophoresis of Mammalian Sera in Starch Gels

SMITHIES[1] has described the electrophoresis of human serum proteins in starch gels. This procedure gives a better resolution of serum proteins than that obtained on paper or in agar gels. The resolution of the serum proteins of cow, pig, horse and dog has been investigated by this method, and it has been found that the pattern obtained is a characteristic of the species.

The procedure and apparatus employed are similar to those of Smithies. The electrophoresis is carried out in boric buffer at 7 V./cm. for 16 hr., the voltage drop being measured between two points 10 cm. apart in the gel. The rear edge of the albumin zone moves 7–9 cm. in this time. If the concentrations of the buffer and bridge solution and the potential are kept constant, the magnitude of the movement is largely governed by the concentration of starch in the gel and the degree of hydrolysis of the starch; these two factors are varied to allow for batch differences in the starch.

Some examples of the electrophoretic patterns obtained are shown in Fig. 1. These diagrams were prepared by projecting an epidiascope image of the stained starch gel on to paper and marking the zones; thus the diagrams do not reflect intensity of staining

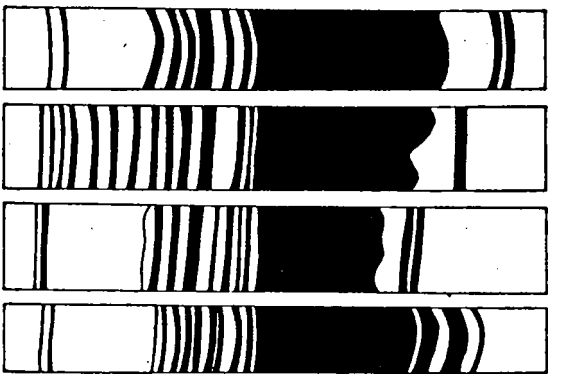


Fig. 1. Epidiascope projections of cow, dog, pig and horse serum (top downwards) after starch gel electrophoresis. Resolution in the albumin band is not shown (see Fig. 2). Arrow indicates direction of separation of protein bands

but merely the position and width of the protein bands. Only the anodic sides of the gels are shown; usually no resolution is apparent on the cathodic side, other than a diffuse γ-globulin band.

Fig. 1 shows that the main points of difference are the number of pre-albumin bands, the total number and position of the post-albumin bands and the presence or absence of a 'discrete' protein band in the pig and some dogs. This latter band stains intensely, is thinner and sharper than the other bands, and is wavy rather than straight.

Under certain electrophoretic conditions, particularly with higher voltage gradients (15–40 V./cm.) and constant current, the post-albumin zones are not well resolved, but the albumin zone shows resolution into three or four bands in the animals examined (Fig. 2). These bands appear to be superimposed on the albumin zone and it seems likely that they are part of the α-globulin complex which migrates at the same rate as albumin, rather than an indication of albumin resolution. With these higher voltage gradients it is necessary to cool the gel during electrophoresis, by supporting it in a 1·5 cm. diameter glass tube within a water jacket.

By increasing the time of hydrolysis of the starch, or alternatively using more acid for a given time, it is possible to prepare hydrolysed starches which gel satisfactorily at 25 per cent w/v. These starches usually have an improved resolving power for the post-albumin bands, but the pre-albumin zones may be lost. It is difficult to compare different gels directly because the bands do not usually coincide, but it has been found that the position of the post-albumin bands relative to the albumin trailing edge (R_{alb} value) is reasonably constant irrespective of distance of migration. The improved resolution of the post-albumin bands from pig sera with more concentrated

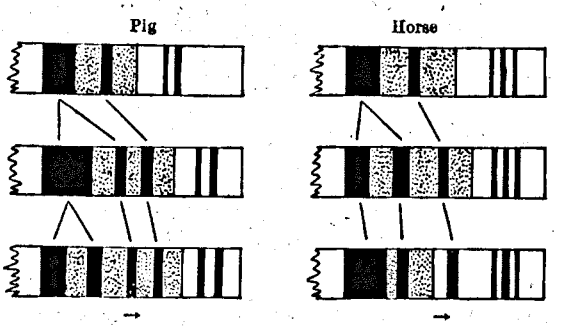
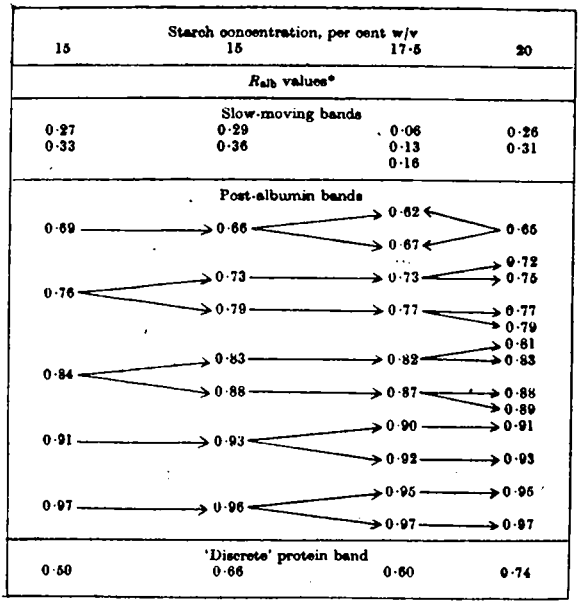


Fig. 2. Diagrammatic illustration of protein bands in albumin zone (albumin stippled). The post-albumin bands are not shown. Arrows indicate direction of separation of protein bands

Table 1



* R_{alb} value = $\frac{\text{distance moved by protein band}}{\text{distance moved by albumin trailing edge}}$

gels is shown in Table 1. The slowest moving group of proteins, and the 'discrete' protein band, if present, are more variable in position.

With the best resolution obtained to date in pigs, it is possible to recognize two pre-albumin bands, an albumin band with four superimposed bands, thirteen immediate post-albumin bands, three slow-moving protein bands, a 'discrete' protein band of variable position and a diffuse γ-globulin band. The characterization of these protein bands in terms of the conventional classification of serum proteins will be the subject of a further communication.

G. C. Ashton

Farm Livestock Research Station,
Animal Health Trust,
Lilystone Hall,
Stock, Essex.
Jan. 15.

[1] Smithies, O., *Biochem. J.*, **61**, 629 (1955).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(Reprinted from Nature, Vol. 182, pp. 945-946. Oct. 4, 1958)

21

Beta-globulin Polymorphism in Cattle, Sheep and Goats

Detection by paper electrophoresis. Using starch gel electrophoresis, six cattle[1], fourteen sheep[2], three goat (see later) and three human[3,4] β-globulin phenotypes have been recognized and indicated to be under the genetic control of three, five, two and three allelomorphs respectively. As one of us had noticed a variation in the number and position of the β-globulin zones in paper electrophoresis of animal sera, the two methods have been compared on sera from cattle, sheep and goats. Starch gel electrophoresis was carried out in phosphate buffer[1] *p*H 7·6, paper electrophoresis on a wide strip of horizontally suspended Whatman 3*MM* paper in barbiturate buffer *p*H 8·6, *I* 0·05. Typical examples of corresponding electropherograms of sheep and cattle sera are shown in Figs. 1 and 2. The electropherograms of goat sera shown in Fig. 3 are discussed below.

It is evident that the different phenotypes in these animals can be distinguished by paper electrophoresis on the basis of β-globulin components of different mobilities. In the simple phenotype system observed in goats (see later), paper electrophoresis was in fact used successfully for independent typing of the sera. From an observation of Smithies[3], it seems likely that human phenotypes could be distinguished on paper also.

Consideration of the electropherograms of homozygous sera shows that each β-globulin zone on paper corresponds to a group of zones in starch-gel electrophoresis, the number of zones in the group depending on the species. In sheep and goats there is a faint zone followed by an intensely staining zone, whereas in cattle there is a faint zone followed by a moderately staining zone and two intensely staining

Table 1. Observed and Expected Distribution of Offspring Phenotypes from Goat β-Globulin Matings

Mating	Offspring β^{AA} Obs.	Exp.	β^{AB} Obs.	Exp.	β^{BB} Obs.	Exp.
$\beta^{AA} \times \beta^{AA}$	No matings					
$\beta^{AA} \times \beta^{AB}$	8	8·5	9	8·5	0	0
$\beta^{AA} \times \beta^{BB}$	0	0	9	9	0	0
$\beta^{AB} \times \beta^{AB}$	1	0·75	1	1·5	1	0·75
$\beta^{AB} \times \beta^{BB}$	0	0	4	5·5	7	5·5
$\beta^{BB} \times \beta^{BB}$	No matings					

Fig. 1. Electropherograms of sheep sera showing β-globulins of sheep sera of four different phenotypes. Left, starch gel; right, paper electrophoresis. Key: α, β and γ = α-, β- and γ-globulins. *alb.*, albumin; *Sa*, slow alpha zone

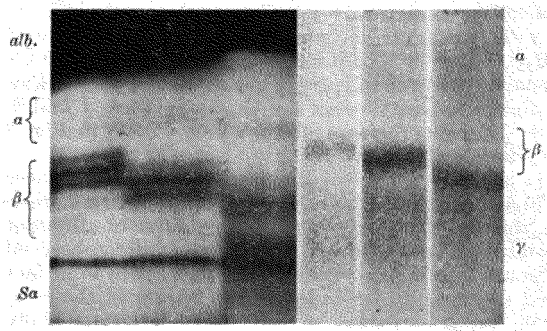


Fig. 2. Electropherograms showing β-globulins of three homozygous cattle sera. Left, starch gel; right, paper electrophoresis. Key: as Fig. 1

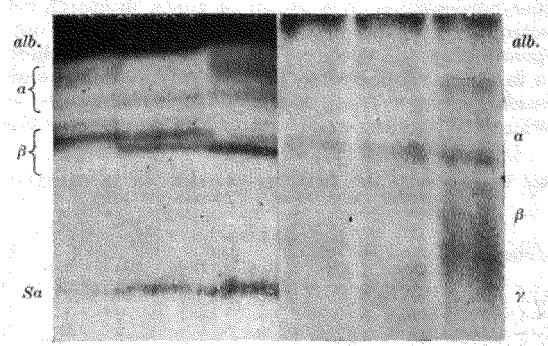


Fig. 3. Electropherograms of goat β-globulin phenotypes. Left, starch gel; right, paper electrophoresis. In each case the phenotypes are from left to right β^{AA}, β^{AB} and β^{BB}. Key: as Fig. 1

zones, described in order of decreasing mobility in the gel.

Comparison of the electropherograms of homozygous and heterozygous sera shows that some of the zones into which the β-globulin components are resolved in starch gel are superimposed in some of the heterozygous sera, particularly in cattle. When this occurs, resolution by paper electrophoresis becomes incomplete and the β-globulins usually appear as a broad zone. This superimposition also makes it difficult to apply a system of nomenclature based on individual coding of the starch gel zones[5] without knowing more about the resolving mechanism and interrelations of the zones in starch gel[6].

β-Globulin polymorphism of goats. Sera from about sixty goats of the British Saanan breed at the Rowett Research Institute have been examined by starch gel electrophoresis using phosphate buffer[1] and by paper electrophoresis. This revealed three phenotypes shown in the electropherograms of Fig. 3. (The two methods are compared above.)

Some animals were examined twice with intervals of up to a year and the phenotype found to be constant. It was also found that mixed sera of the two outermost phenotypes shown in Fig. 3 were indistinguishable from the third (centre) when run together in starch gel. This suggested that the β-globulins of goats are controlled by two allelomorphs β^A and β^B, giving rise to the genotypes β^A/β^A, β^A/β^B and β^B/β^B, and the three phenotypes β^{AA}, β^{AB} and β^{BB}. This hypothesis is supported by mating results shown in Table 1, where the observed and expected distribution of phenotypes of some forty offspring are compared.

This genetic mechanism is similar to that proposed for β-globulin polymorphism in cattle, sheep and man.

G. C. Ashton*

The Animal Health Trust
Farm Livestock Research Centre,
Lilystone Hall,
Stock,
Essex.

E. I. McDougall

The Rowett Research Institute,
Bucksburn,
Aberdeenshire.
July 3.

* Present address: Sheep Biology Station, P.O. Box 144, Parramata, N.S.W., Australia.

[1] Ashton, G. C., *Nature*, **180**, 917 (1957); *ibid.* (in the press).
[2] Ashton, G. C., *Nature*, **181**, 849 (1958); *ibid.* (in the press).
[3] Smithies, O., *Nature*, **180**, 1482 (1957).
[4] Smithies, O., *Nature*, **181**, 1203 (1958).
[5] Poulik, M. D., and Smithies, O., *Biochem. J.*, **68**, 638 (1958).
[6] Franglen, G., and Gosselin, C., *Nature*, **181**, 1152 (1958).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(*Reprinted from Nature*, *Vol.* 181, *pp.* 849–850, *March* 22, 1958)

Polymorphism in the β-Globulins of Sheep

POLYMORPHISM in the β-globulins of cattle, giving five phenotypes[1,2], and in humans giving three phenotypes (Smithies, O., personal communication), has been described. The present communication concerns polymorphism in the β-globulins of the serum of sheep, giving to date eight phenotypes. The phenotypes are indicated diagrammatically in Fig. 1. The conditions for obtaining these serum protein differences by starch gel electrophoresis are similar to those described for cattle sera[1] except that 5–6 hr. is required to achieve adequate resolution of the β-globulins; in this time the trailing edge of the albumin zone migrates 6–7 cm.

It seems probable that the greatest degree of polymorphism in the serum proteins of mammals will be found in the β-globulins, notwithstanding the fact

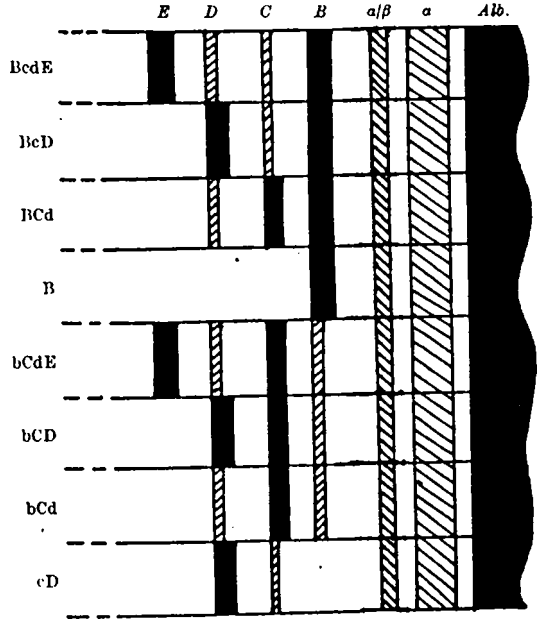


Fig. 1. Diagrammatic representation of β-globulin polymorphism in sheep sera by one-dimensional starch gel electrophoresis. Only a portion of the anodic side of the gel is shown. *B*, *C*, *D* and *E*, β-globulins; *a*/β, zone consisting of β-globulin *A* plus some α-globulin components; *a*, α-globulin components; *alb.*, part of albumin zone. Solid bands, intense staining; cross-hatched, medium or faint staining. For phenotype code, see text

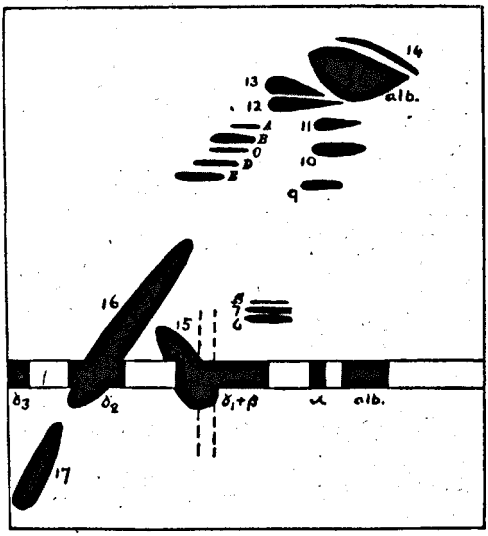


Fig. 2. Diagram prepared after two-dimensional agar/starch gel electrophoresis of type BcdE serum. Broken lines indicate position of original sample insertion in agar. *Alb.*, albumin; *α*, α-globulin (zones 9–13); *β*, β-globulin (zones *A*, *B*, *C*, *D*, *E*); γ_1, γ_2, γ_3, γ-globulins (zones 15, 16 and 17); zones 6, 7, 8, 'slow α-globulins'; zone 14, pre-albumin

that serum protein polymorphism was first detected by Smithies in the haptoglobins of humans[3]. A rational nomenclature for the β-globulin phenotypes, sufficiently flexible to cover analogous systems in other animals, is therefore necessary. It is suggested that the proteins contributing to each β-globulin polymorphic system be designated by letters in sequence starting at the β-globulin nearest the albumin, that is, the one with greatest mobility; an intensely staining zone could be designated by a capital letter, a less intensely staining zone by a lower case letter, and the absence of the zone by omission of the letter. Such a phenotype nomenclature which makes no implications about the genotypes, is shown for the sheep β-globulin types in Fig. 1.

The distribution of the eight phenotypes in sheep found by examination of 118 abattoir samples (mixed breeds) was: 17 BcdE, 18 BcD, 13 BCd, 5 B, 2 bCdE, 45 bCD, 3 bCd and 15 cD.

The nature of the zones contributing to the one-dimensional starch gel electrophoretic patterns in sheep was determined by two-dimensional agar-starch gel electrophoresis as used for cattle[1] (Fig. 2).

It will be seen that the β-globulins coincide with some of the α-globulin components when the sera

are run in starch gel only. Fortunately the α-globulins do not stain sufficiently intensely to obscure the β-globulin pattern, although where β-globulin *B* is absent, α-globulin zones 10 and 11 (Fig. 2) can be seen as background staining. Usually the α-globulin zone 9 (Fig. 2) can be seen as a faint poorly defined zone just behind β-globulin *E*. No 'thread proteins' such as those found in cattle[1] or pigs[4] were observed in sheep.

So far insufficient mating data have been obtained to postulate genetic control, but it seems probable, by analogy with cattle and human results, that the various phenotypes will prove to be inherited.

G. C. ASHTON

The Animal Health Trust,
Farm Livestock Research Centre,
Stock, Essex.
Jan. 9.

[1] Ashton, G. C., *Nature*, **180**, 917 (1957).
[2] Smithies, O., and Hickman, C. G., *Genetics* (in the press).
[3] Smithies, O., and Walker, N. F., *Nature*, **178**, 694 (1956).
[4] Ashton, G. C., *Nature*, **179**, 824 (1957).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(*Reprinted from Nature, Vol.* 182, *pp.* 1101–1102, *Oct.* 18, 1958)

Further β-Globulin Phenotypes in Sheep

In a preliminary communication eight sheep β-globulin types were described[1]. This number has now been increased partly by recognition of the fact that the two strongly staining zones *C* and *D* can be resolved into a third zone, and partly by the discovery of further types during examination of five hundred samples of sheep serum from Chelmsford abattoir.

The fourteen phenotypes which have been found so far (Fig. 1) probably represent the ten heterozy-

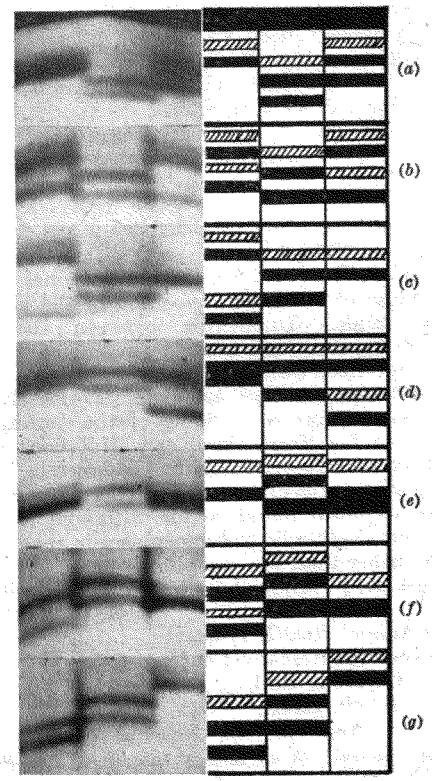


Fig. 1. Fourteen sheep *β*-globulin phenotypes. The illustration shows the *β*-globulin region of seven starch-gel electropherograms, each gel carrying three sera. The phenotypes are, from left to right on each gel, (*a*) βAA, βBD, βAB; (*b*) βAC, βBD, βAD; (*c*) βAE, βBD, βBB; (*d*) βBC, βBD, βBE; (*e*) βCC, βBD, βCD; (*f*) βCE, βBD, βDD; (*g*) βDE, βBD, βAA. The trailing edge of the albumin zone is shown at the top of gel (*a*) for orientation

Table 1. β-GLOBULIN MATING RESULTS FROM EWES MATED WITH A β^{BB} RAM

Mating	Phenotype of offspring				
	AB	*BB*	*BC*	*BD*	*BE*
BB × *AB**	2	2	—	—	—
BB × *AD*	0	—	—	1	—
BB × *AE*	0	—	—	—	2
BB × *BB*	—	1	—	—	—
BB × *BC*	—	5	2	—	—
BB × *BD*	—	0	—	2	—
BB × *BE*	—	0	—	—	1
BB × *CC*	—	—	1	—	—
BB × *CD*	—	—	2	5	—
BB × *CE*	—	—	2	—	0
BB × *DD*	—	—	—	1	—

* *AB* is equivalent to β^{AB} and so on.

gotes, and four of the five homozygotes of five β-globulin alleles, sheep β^A, β^B, β^C, β^D and β^E.

In keeping with the phenotype nomenclature suggested for the cattle[1] and goat[2] β-globulin systems. the phenotypes have been named sheep β^{AA}, β^{AB} . . . β^{EE}, representing the individual genotypes β^A/β^A, β^A/β^B, . . . β^E/β^E. So far the homozygote β^{EE} has not been found.

Each allele produces two zones in starch gel, an intensely staining zone preceded by a less intensely staining zone[3] (Fig. 1). In types involving combinations of β^B, β^C and β^D, the fainter zones are not seen because of the superimposition of the intensely staining zones. Distinction between the sheep β-globulin types is best made by reference to a β^{BD} serum run on the same gel.

120 different matings are possible with the five-allele system proposed. Some of the mating data obtained so far (from the Kerry Hill flock at the Farm Livestock Research Centre) is shown in Table 1.

Sheep exhibit the most complex example of β-globulin polymorphism found so far, although it is now known that β-globulin polymorphism in mammals is the rule rather than the exception.

I thank K. F. Mitchell for his technical assistance.

G. C. ASHTON*

The Animal Health Trust,
Farm Livestock Research Centre,
Lilystone Hall,
Stock, Essex.

Aug. 1.

* Present address: Sheep Biology Laboratory, P.O. Box 144, Parramatta, N.S.W.

[1] Ashton, G. C., *Nature*, 181, 849 (1958).
[2] Ashton, G. C., *Nature*, 182, 370 (1958).
[3] Ashton, G. C., and McDougall, E. I., *Nature* (in the press).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Commonwealth Scientific and Industrial Research Organization
Reprinted from "Genetical Research" Vol. 4 No. 2, *pp.* 240–247
Printed in Great Britain

Serum transferrins in Merino sheep

By G. C. ASHTON
Cattle Research Laboratory, C.S.I.R.O., Rockhampton, Queensland, Australia

AND

K. A. FERGUSON
Ian Clunies Ross Animal Research Laboratory, C.S.I.R.O., Prospect, New South Wales, Australia

(*Received* 14 *September* 1962)

1. INTRODUCTION

Polymorphism of the iron-binding β-globulins, also called transferrins and siderophilins, has been described in numerous mammalian species, including man (Smithies, 1957), cattle (Ashton, 1957; Hickman & Smithies, 1957), sheep (Ashton, 1958*a*), goats (Ashton & McDougall, 1958; Millson & Pattison, 1961), horses (Ashton, 1958*b*), pigs (Ashton, 1960*a*; Kristjansson, 1960), mice (Ashton & Braden, 1961; Cohen, 1961; Shreffler, 1961), chimpanzees (Boyer & Young, 1960; Buettner-Janusch, 1961), monkeys (Lai & Kirk, 1960; Blumberg, 1960; Goodman & Poulik, 1961), reindeer (Gahne & Rendel, 1961), and deer (Lowe & McDougall, 1961). In each species the genetic mechanism, where established, has proved to be essentially the same. The polymorphism is due to multiple autosomal allelomorphs exhibiting co-dominance, each allele causing the production of two or more protein staining zones in starch-gel after electrophoresis. The number and relative staining intensity of the zones produced by an allele are a characteristic of the species. In cattle and buffalo (Ashton, Jenkins & Tulloch, unpublished data) each allele produces four zones. Three zones are produced for each allele in pigs, mice, reindeer and horses. Two zones are produced in humans, chimpanzees, sheep and goats. No case is known where only one zone is formed. The reasons for this multiplicity of zones from one allele has been discussed by various authors (e.g. Ashton & Braden, 1961; Cohen, 1961; Patras & Stone, 1961).

English breeds of sheep examined previously (Ashton, 1958*a*) have shown five β-glubulin alleles. Staining with nitroso-R salt has shown that these are iron-binding proteins and hence transferrins. Examination of merino sheep has shown additional transferrins which are described in this paper.

2. MATERIALS AND METHODS

(i) *Starch gel electrophoresis*

For routine examination the serum proteins of sheep have been examined in an apparatus described elsewhere (Ashton, 1957, 1960*b*), which is a simple modification

of that described originally by Smithies, (1955). A discontinuous tris-citric buffer system (Poulik, 1957) as modified by Ferguson & Wallace (1961) was employed.

Some sera were also examined under high-voltage conditions in an apparatus which will be described elsewhere (Ferguson, unpublished data). The separation of transferrin types illustrated in Plate I was obtained with this apparatus.

Samples of serum for typing were run on the same gel as one or more reference samples of defined transferrin type.

(ii) *Serum samples*

Serum samples were obtained from four main sources:

(i) 225 samples from Tasmania from two of the original studs (Kenilworth and Valleyfield) on which the Tasmanian fine-woolled Merino was founded;

(ii) 1356 samples from Merinos, and 'Merinos' with some Border Leicester ancestry, at the National Field Station, 'Gilruth Plains', Cunnamulla, Queensland;

(iii) samples from the F. D. McMaster Field Station, Badgery's Creek, N.S.W., as follows: sera from thirty-six ewes purchased from the Pepping commercial flock used to establish the Badgery's Creek inbred families; samples from thirty-five animals from inbred sires and unrelated ewes of mixed Peppin origin; and samples from thirteen ewes from two of the inbred families;

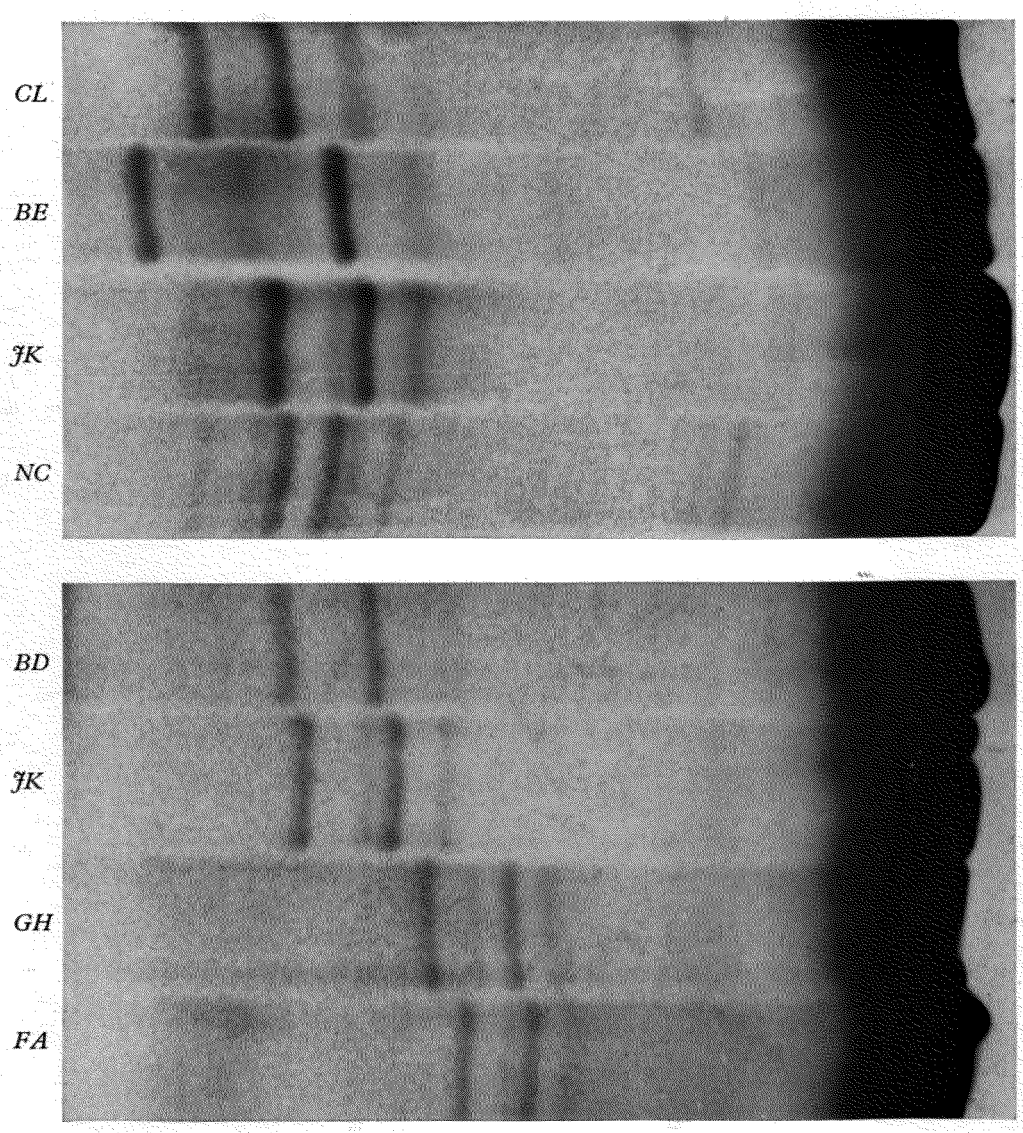
(iv) 298 samples from a stud flock of Pepping Merinos in Western Central Queensland.

(iii) *Nomenclature of transferrin phenotypes*

The nomenclature of transferrin polymorphism is complicated by the multiple zones produced by each allele and by the ever-increasing number of newly recognized alleles in all species examined.

The fact that a transferrin allele gives rise to more than one zone in starch gel was first reported by Ashton & McDougall (1958) from a comparison of paper and starch gel electrophoretic results with cattle, sheep and goat sera. To facilitate description of newly discovered phenotypes in sheep the term 'zone pair' has been used to describe the two zones produced in starch-gel by a sheep transferrin allele. Any new allele can then be defined in terms of the mobility of the zone pair it produces in starch-gel under the stated conditions, relative to established reference sera. (In the same way species with alleles producing three zones might be considered to have transferrin zone trios, and species with four zones, transferrin zone quartets.)

The naming of newly-discovered alleles presents the main problem. This has been discussed by Cohen & Shreffler (1961) with particular reference to the mouse transferrin locus, but their remarks are applicable to other species and other multiple allelomorphic systems. Cohen & Shreffler state, 'If new electrophoretic types of transferrin are found these should be designated alphabetically in order of discovery. If the same letter should happen to be assigned to two different types these might be distinguished by prefacing the duplicated letter with another,



Starch gel electrophoresis of transferrin *A* in sheep sera. 17 V/cm for 21 hours. 90% 0·0033 M citric acid, 0·025 M tris (hydroxymethyl) aminomethane, 10% 0·02 M lithium hydroxide, 0·076 M boric acid in gel. 0·1 M lithium hydroxide, 0·38 M boric acid in electrode vessels.

designating for example the laboratory which reported it.' This system has been used in naming the newly discovered alleles reported in this paper.

The locus symbol for the polymorphic β-globulins was originally 'β'. Following the discovery (Smithies & Hiller, 1959; Giblett, Hickman & Smithies, 1959) that these β-globulins represented the iron-binding protein transferrin or siderophilin in human and cattle serum these authors adopted the locus symbol 'Tf' as more descriptive than 'β'. The locus symbol 'Tf' has been adopted in this publication also, following the observation that both proteins of the β-globulin zone pair in sheep bind iron.

3. RESULTS

(i) *Newly-detected alleles*

A survey of Merino serum samples showed the presence of a considerable number of phenotypes which had not been seen in sheep serum samples examined in England (Ashton, 1958*a*). Fourteen out of the fifteen possible types from five alleles had previously been seen and the alleles had been defined as sheep β^A, β^B, β^C, β^D and β^E according to decreasing order of mobility of the transferrin zone pairs in starch-gel. The fifteenth type *TfEE* has now been found (Sellers & Mitchell, personal communication).

Serum samples of known genotype, originating from the laboratory where the sheep transferrin types were first defined were compared with the Merino serum samples listed above. A number of previously unrecognized phenotypes were found. By comparison with the reference samples, and knowing that each allele in sheep produces a zone pair consisting of a faster moderately staining and a slower more intensely staining zone, it was possible to identify seven additional sheep transferrin alleles as follows:

(*a*) Two alleles producing zone pairs migrating more rapidly than that from Tf^A. These have been called TF^F and Tf^G, Tf^F producing the fastest transferrin zone pair found so far in sheep sera.

(*b*) Two alleles producing zone pairs intermediate in mobility between those produced by Tf^A and Tf^B. These alleles have been called Tf^H and Tf^J, Tf^H producing the faster zone pair.

(*c*) An allele producing a zone pair between those produced by Tf^C and Tf^D. This allele has been called Tf^K.

(*d*) An allele producing a zone pair between those produced by Tf^D and Tf^E. which has been called Tf^L.

(*e*) An allele producing a zone pair between those produced by Tf^B and Tf^C. This allele has been called Tf^N.

The alleles have been coded in the order in which they were recognized, and not in order of decreasing mobility. The relative position of the twelve defined sheep transferrin alleles are shown in Fig. 1 and examples of phenotypes produced by these alleles are shown in Plate I.

(*a*) Tf^F *and* Tf^G

Zone pairs migrating more rapidly than that produced by Tf^A occurred frequently in the first samples examined. Subsequently it was realized that two zone pairs were present, with only slightly different mobilities. The faster zone pair was defined as due to the allele Tf^F which is quite common in Merinos, and the slower to Tf^G which is rather infrequent. It was found advisable to re-run all samples with, Tf^F or Tf^G represented to verify the phenotype.

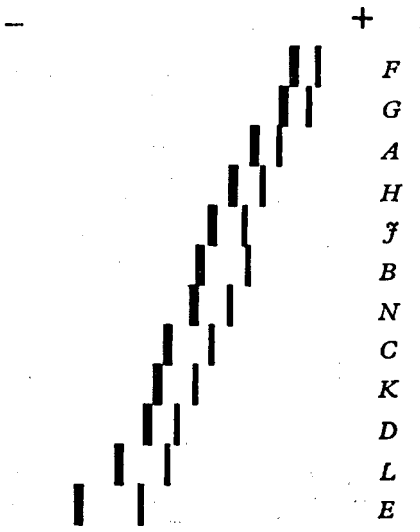


Fig. 1. Relative mobility of transferrin types in sheep sera by starch gel electrophoresis. Electrophoretic conditions as described in Plate 1.

(*b*) Tf^H *and* Tf^J

The zone pairs produced by Tf^H and Tf^J are well-resolved from each other and from Tf^A and Tf^B. Tf^H and Tf^J alleles are frequent in Merinos.

(*c*) Tf^K, Tf^L, Tf^N

Only a small number of phenotypes produced by these newly discovered alleles were found. Several hundred samples were examined before a Tf^N heterozygote was found. The homozygote, Tf^L/Tf^L has been found, but not the homozygotes Tf^K/Tf^K or Tf^N/Tf^N.

The zone pairs produced by Tf^K, Tf^L, and Tf^N are readily distinguishable from the zone pairs preceding or succeeding them in starch gel.

(*d*) Tf^A *and* Tf^C

Tf^A was fairly frequent and Tf^C infrequent in the serum samples examined.

(*e*) Tf^B, Tf^D, *and* Tf^E

Zone pairs due to these alleles were not found in the Merino serum samples examined in this survey.

(ii) TfDD *reference samples*

The zone pairs due to Tf^D in the heterozygous English reference sera *TfAD*, *TfBD*, *TfCD* and *TfDE* had the same electrophoretic mobility. Using these sera Tf^K and Tf^L were defined as alleles producing zone pairs migrating faster (Tf^K) and slower (Tf^L) than that produced by Tf^D.

Two examples of nominal *TfDD* reference sera were also available. On examination in the discontinuous tris buffer system these samples proved to be heterozygotes, one being *TfDL* and the other *TfKL*. Apart from illustrating the markedly improved resolution achievable with the discontinuous buffer system, these two samples show that Tf^K and Tf^L occur in British breeds of sheep, probably with reasonable frequency.

(ii) *Deterioration of reference samples*

The definition of new alleles depends on the comparison of a serum showing a suspected new phenotype with reference sera. It is important therefore that the reference sera should not have changed in electrophoretic mobility prior to the comparison.

The effect of heat (warming in a water bath at 50° C) and bacterial contamination (exposure to laboratory atmosphere) on the mobility of transferrin zone pairs of several sera of known phenotype was examined. In each case both treatments eventually produced a phenotype with double the number of zone pairs originally present. In every case the newly formed zone pairs were slower in mobility than the original. On prolonged treatment the zones became diffuse and eventually unrecognizable.

This behaviour is similar to that found with red deer phenotypes by Lowe & McDougall (1961). It may be due to the splitting-off of sialic acid from the transferrins by neuraminidase (Poulik, 1959; Blumberg & Warren, 1961).

None of the English reference sera, which have been stored continuously at −17° C. for four years, has shown any signs of such deterioration.

(iv) *Mating results*

No exceptions have been found, in a variety of animal species examined, to the hypothesis that transferrin polymorphism in each species is controlled by a multiple autosomal allelomorphic system exhibiting co-dominance. A relatively small number of mating results in English sheep (Ashton 1958*a*) fitted such a hypothesis. The distributions of 177 offspring from Merino ewes and rams, involving the transferrin alleles *F*, *G*, *A*, *H*, *J* and *K*, were in accord with the hypothesis.

Extensive mating data are being collected, in co-operation with Miss H. N. Turner and Mr G. H. S. Dolling, to test for fertility effects similar to those found in cattle (Ashton, 1961; Ashton & Fallon, 1962) and will be reported later.

(v) *Distribution of alleles in flocks examined*

Tf^F, Tf^A, Tf^H, Tf^J and Tf^K were present in all four flocks. Tf^G was present in only two of the four flocks, namely in those from Western Central Queensland and

the F. D. McMaster Field Station. Tf^L and Tf^N were found in low frequency, and only in the flock at the National Field Station. Out of 1356 sheep sampled there were two Tf^L homozyogtes and seventeen Tf^L heterozygotes. No Tf^N homogyzotes were found, but there were thirteen Tf^N heterozygotes. The National Field Station flock was the only flock in which Tf^C was found. There were five Tf^C homozygotes and thirty-eight heterozygotes. It is not yet known if Tf^C was present because of the Border-Leicester ancestry of some of the sheep sampled.

Because the National Field Station flock has been subjected to strong selection pressures for different factors, it is unlikely that gene frequencies found for the 1356 sheep sampled would be representative of Merinos in general. The gene frequencies for the other three flocks are shown in Table 1, but no attempt was made to ensure that the samples taken were representative of the flocks in the area from which they were drawn.

Table 1. *Transferrin gene frequencies and standard errors for various sheep populations studied*

Population	No. of animals	Tf^F	Tf^G	Tf^A	Tf^H	Tf^J	Tf^K
Tasmanian fine-woolled Merinos	210	0·012 ±0·005	—	0·314 ±0·023	0·279 ±0·019	0·371 ±0·028	0·024 ±0·007
Peppin Merinos from Central Western Queensland	298	0·181 ±0·016	0·059 ±0·010	0·149 ±0·015	0·200 ±0·016	0·391 ±0·020	0·020 ±0·006
Peppin Merinos from Badgery's Creek, N.S.W.	71*	0·112 ±0·037	0·084 ±0·033	0·075 ±0·031	0·187 ±0·046	0·458 ±0·059	0·084 ±0·033

* Excluding inbred animals, and counting maternally bestowed genes only in the group of offspring from ewes mated to inbred rams.

4. DISCUSSION

The recognition of a further seven transferring alleles in sheep, brings the number of described alleles to twelve. Sellers & Mitchell (personal communication) have found a phenotype with a zone pair migrating more slowly than that produced by Tf^E in a sample from a Derbyshire Gritstone sheep. These authors have also seen phenotypes with zone pairs migrating faster than that produced by Tf^A. It seems probable that these represent Tf^F or Tf^G or both. Direct comparison between samples will be necessary to define similarities or differences in mobility.

There are clearly marked breed differences in the type of genes represented, and the frequency of these genes, between the British breeds examined previously (Kerry Hill, Welsh Mountain, and Lincoln) and the Australian Merino. Also there may be within-breed differences between Tasmanian fine-woolled and Queensland Peppin Merinos (Table 1). Further and more strictly controlled sampling will be necessary to establish between- and within-breed gene frequencies.

There are probably at least thirteen transferrin alleles in English and Merino sheep. Examination of other breeds from other parts of the world may well reveal further alleles. Polymorphism is considered to be a means whereby a species is able to adapt to varying environments and ecological situations. As such polymorphism, and particularly biochemical polymorphism, is worthy of study by animal breeders concerned with choosing the right type of animal for a given location. The widespread geographical occurrence of sheep may be a reflection of their high level of biochemical polymorphism, among which the serum transferrins may be important.

Two ways in which transferrin polymorphism may influence the adaptability of sheep breeds may be inferred from results obtained in cattle. It has been shown that milk yield of dairy cattle is correlated with transferrin type (Ashton, 1960*b*; Ashton, Fallon & Sutherland, 1962). If a similar association occurs in sheep the growth rate of suckling lambs, and hence their chance of survival, wool production, etc., may be partly related to maternal transferrin type. It must be emphasised, however, that such effects would be detected only by determining the average values (milk yield, birth-weaning gain, etc.) of a large random group of animals, and would have no value in predicting, for example, the milk yield of an individual.

It has also been shown that parental transferrin type affects the fertility of artificially inseminated dairy cattle (Ashton, 1961; Ashton & Fallon, 1962). Thus matings between homozygotes proved very significantly more fertile than matings involving heterozygotes. If the parental β-globulin type in sheep is found to influence fertility, as in cattle, then this might have practical significance in increasing conception rates in artificial insemination.

5. SUMMARY

Serum samples from 1963 Merino sheep were examined for serum transferrin type. Two of the five transferrin alleles previously described in British breeds of sheep, viz. Tf^A and Tf^C, were found, but Tf^B, Tf^D and Tf^E were absent. Evidence for seven further transferrin alleles was obtained. These alleles were coded Tf^F, Tf^G, Tf^H, Tf^J, Tf^N, Tf^K and Tf^L in decreasing order of mobility of the zones they produce in starch gel.

Gene frequency data is presented for the populations studied.

Blood samples and records from Tasmania were kindly supplied by Mr P. M. Houlahan of the Department of Agriculture, Launceston, and from the McMaster Field Station, Badgery's Creek, N.S.W., by Mr R. Hayman, Officer-in-Charge. We are grateful to Miss H. N. Turner, Mr G. H. S. Dolling, and Professor J. V. Evans for making available blood serum samples from the National Field Station, 'Gilruth Plains', Cunnamulla, Queensland, and allowing us to present gene frequency data from this co-operative project; and to Dr G. R. Moule for inviting us to examine serum samples from sheep in one of his experiments in Western Central Queensland.

REFERENCES

Ashton, G. C. (1957). Serum protein differences in cattle by starch gel electrophoresis. *Nature, Lond.*, **180**, 917–919.

Ashton, G. C. (1958*a*). Further β-lgobulin phenotypes in sheep. *Nature, Lond.*, **182**, 1101–1102.

ASHTON, G. C. (1958*b*). Serum protein variations in horses. *Nature, Lond.*, **182**, 1029–1030.
ASHTON, G. C. (1960*a*). Thread protein and β-globulin polymorphism in the serum proteins of pigs. *Nature, Lond.*, **186**, 991–992.
ASHTON, G. C. (1960*b*). β-globulin polymorphism and economic factors in dairy cattle. *J. agric. Sci.* **54**, 321–328.
ASHTON, G. C. (1961). β-globulin type and fertility in artificially bred dairy cattle. *J. Reprod. Fert.* **2**, 117–129.
ASHTON, G. C. & BRADEN, A. W. H. (1961). Serum β-globulin polymorphism in mice. *Aust. J. biol. Sci.* **14**, 248–253.
ASHTON, G. C. & FALLON, G. R. (1962). β-globulin type fertility, and embryonic mortality in cattle. *J. Reprod. Fert.* **3**, 93–104.
ASHTON, G. C., FALLON, G. R. & SUTHERLAND, D. O. (1962). β-globulin (transferrin) type and milk and butterfat production in dairy cows. *J. agric. Sci.* (In the press.)
ASHTON, G. C. & MCDOUGALL, E. I. (1958). β-globulin polymorphism in cattle, sheep and goats. *Nature, Lond.*, **183**, 945–946.
BLUMBERG, B. S. (1960). Biochemical polymorphisms in animals: Haptoglobins and transferrins. *Proc. Soc. exp. Biol. N.Y.* **104**, 25–28.
BLUMBERG, B. S. & WARREN, L. (1961). *Biochem. biophys. Acta*, **50**, 90.
BOYER, S. H. & YOUNG, W. J. (1960). β-globulin polymorphism in chimpanzees. *Nature, Lond.*, **187**, 1035–1036.
BUETTNER-JANUSCH, J. (1961). Transferrin differences in chimpanzee sera. *Nature, Lond.*, **192**, 632–633.
COHEN, B. L. (1960). Genetics of plasma transferrin in the mouse. *Genet. Res.* **1**, 431–438.
COHEN, B. L. & SHREFFLER, D. C. (1961). A revised nomenclature for the mouse transferrin locus. *Genet. Res.* **2**, 306–308.
FERGUSON, K. A. & WALLACE, A. L. C. (1961). Starch-gel electrophoresis of anterior pituitary hormones. *Nature, Lond.*, **190**, 629–630.
GAHNE, B. & RENDEL, J. (1961). Blood and serum groups in reindeer compared with those in cattle. *Nature, Lond.*, **192**, 529–530.
GIBLETT, E. R., HICKMAN, C. G. & SMITHIES, O. (1959). Serum transferrins. *Nature, Lond.*, **183**, 1589–1590.
GOODMAN, M. & POULIK, E. (1961). Serum transferrins in the genus *Macaca*: Species distribution of nineteen phenotypes. *Nature, Lond.*, **191**, 1407–1408.
HICKMAN, C. G. & SMITHIES, O. (1957). Evidence for inherited differences in the serum proteins of cattle. *Proc. gen. Soc. Can.* **2**, 39.
KRISTJANSSON, F. K. (1960). Inheritance of a serum protein in swine. *Science*, **131**, 1681.
LAI, L. Y. C. & KIRK, R. L. (1960). β-globulin variants in two species of monkeys. *Nature, Lond.*, **188**, 673–674.
LOWE, V. A. W. & MCDOUGALL, E. I. (1961). Serum β-globulin types in red deer and other species and their stability in the presence of bacteria. *Nature, Lond.*, **192**, 983–984.
MILLSON, G. C. & PATTISON, I. H. (1961). β-globulin polymorphism in goats. *Vet. Rec.* **73**, 256.
PATRAS, B. & STONE, W. H. (1961). Partial purification of cattle serum transferrin using rivanol. *Proc. Soc. exp. Biol., N.Y.*, **107**, 861–864.
POULIK, M. D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. *Nature, Lond.*, **180**, 1477–1479.
POULIK, M. D. (1959). Starch gel immuno-electrophoresis. *J. Immunol.* **82**, 502–515.
SHREFFLER, D. C. (1960). Genetic control of serum transferrin type in mice. *Proc. nat. Acad. Sci., Wash.*, **46**, 1378–1384.
SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629–641.
SMITHIES, O. (1957). Variants in human serum β-globulins. *Nature, Lond.*, **180**, 1482–1483.
SMITHIES, O. & HILLER, O. (1959). The genetic control of transferrins in humans. *Biochem. J.* **72**, 121–126.

(*Reprinted from Nature, Vol.* 182, *pp.* 1029–1030, *Oct.* 11, 1958)

Serum Protein Variations in Horses

IT is now clear that intraspecific serum protein variation is widespread in mammals at least, and that the technique of starch-gel electrophoresis developed by Smithies[1] is a powerful tool for revealing these differences. Complex protein patterns have been obtained with sera from horses, due to concomitant variation in several protein systems.

Sera from 79 thoroughbred and other ('knacker') horses of unspecified breed were examined, using *p*H 7·6 phosphate buffer and electrolyte as described previously[2]. Four variable systems were found, namely, the β-globulins, pre-albumins and two apparently unrelated α-globulin systems. Electropherograms of three sera illustrating these variations are shown in Fig. 1. A diagram of a two-dimensional agar/starch-gel electropherogram[3] of another serum is shown in Fig. 2.

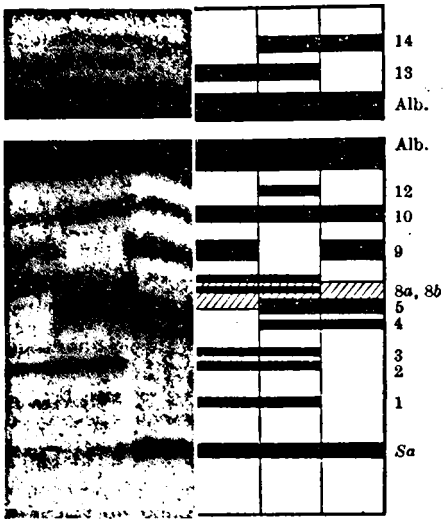


Fig. 1. Starch gel electrophoresis protein patterns from three representative horse sera. Only the anodic side of the gel is illustrated. The main part of the gel was from a 5-hr. run at 200 V.; the pre-albumin portion was from a separate 3-hr. run at the same voltage. Alb., albumin; *Sa*, slow-alpha globulins; 1–6, β-globulins; 8*a*, 8*b* and 9, first variable α-globulins; 12, second variable α-globulin; 13 and 14, pre-albumins. Zone 7 (cross-hatched) is usually rather diffuse and occurs in the region of zones 5, 6 and 8

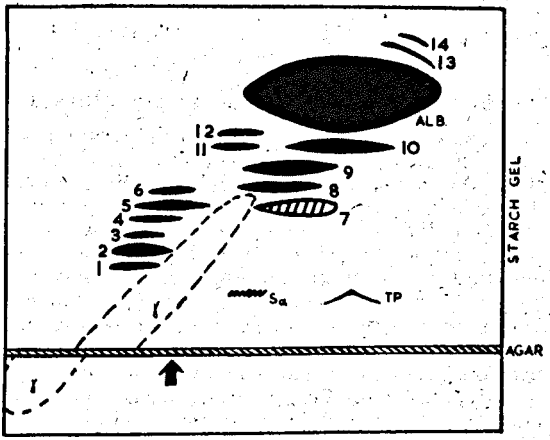


Fig. 2. Diagram of a two-dimensional electropherogram of a selected horse serum. The arrow indicates the position of insertion of the original sample in the agar. γ, γ-globulins; *TP*, thread protein. See also key to Fig. 1

Three β-globulin types were observed. It is possible that these represent the homozygotes and heterozygote of two β-globulin alleles, one producing zones 1, 2 and 3 and the other zones 4, 5 and 6. This would be comparable with previously described β-globulin systems in other species[3].

Three types have been found in the first of the variable α-globulin systems depending on the presence of either or both zones 8 and 9. In some gels zone 8 is resolved into two zones, 8*a* and 8*b*, but neither of these has so far been found alone. The α-globulin zone 7 is apparently always present but is not sharply defined. In one-dimensional runs it appears in the region of zones 5, 6 and 8 as a diffuse band. Experiments with added hæmoglobin suggest that this may be the hæmoglobin-binding protein (haptoglobin).

The second variable α-globulin system has shown two types depending on the presence or absence of zone 12. The distinction between these types is not always easy in one-dimensional runs, but is made more readily in two-dimensional separations.

At least three pre-albumin types have been observed in the sera examined, depending on the presence of either or both zones 13 and 14. However, a third pre-albumin zone, intermediate in mobility to the other two, has been seen in two sera, but only in the absence of zone 14.

It will be necessary to examine an appropriate number of related individuals to determine if these variations are inherited, that is, constitute further examples of serum protein polymorphism.

I thank Mr. A. Thomson, of the Wellcome Research Laboratories, and the staff of the Equine Research Station of the Animal Health Trust for providing horse sera, and K. F. Mitchell for technical assistance.

G. C. ASHTON*

The Animal Health Trust,
Farm Livestock Research Centre,
Lilystone Hall,
Stock, Essex. July 25.

* Present address: Sheep Biology Laboratory, P.O. Box 144, Parramatta, N.S.W., Australia.

[1] Smithies, O., *Biochem. J.*, **61**, 629 (1955).
[2] Ashton, G. C., *Nature*, **180**, 917 (1957).
[3] Ashton, G. C., and McDougall, E. I., *Nature*, [182, 945 (1958)].

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(26)

(Reprinted from Nature, Vol. 186, No. 4729, pp. 991–992, June 18, 1960)

Thread Protein and β-Globulin Polymorphism in the Serum Proteins of Pigs

THREAD proteins, reported to be polymorphic in the sera of cattle[1], have now been demonstrated in serum samples from pigs, dogs, and horses. Thread protein polymorphism has been found in the pig also, but not in the relatively few samples from dogs and horses examined up to the present.

Pig thread proteins are readily seen in one-dimensional starch-gel electropherograms using the same *p*H 7·6 phosphate buffer and procedure described for cattle sera[2]. The zones are very narrow, indeed thread-like, in suitable gels. However, the zones may be faint, or not appear at all if the nature of the gel deviates only a little from the optimum. No difficulty has been experienced in detecting thread proteins in gels prepared from several batches of Hopkin and Williams potato starch after suitable acid hydrolysis[3]; but it has not been possible to detect thread proteins, particularly in cattle sera, using a commercially available starch hydrolysed ready for starch-gel electrophoresis.

Three thread-protein phenotypes have been found in sera from Large White and Essex Saddleback pigs, depending on the presence of one or both of a pair of thread proteins. The three phenotypes are shown in Fig. 1, after two-dimensional electrophoresis, first in agar then in starch gel. Sera of the three phenotypes were resolved initially in 1 per cent agar using *p*H 8·4 borate buffer (4·65 gm. boric acid in water adjusted with sodium hydroxide and diluted to 1 l.). Afterwards 0·5 cm. wide length-wise strips of agar containing the resolved albumin zone were cut from each of the three agar gels and inserted side-by-side across a previously prepared starch gel of suitable width. After electrophoresis the starch gel was sliced with fine wire and stained in nigrosine[3]. Fig. 1 shows that the agar albumin zones also contained some α-globulins, the thread proteins and a pre-albumin zone.

The nature of the thread proteins is not clear. In all animals in which they have been found they occupy the same relative position after two-dimensional electrophoresis, that is, at the leading edge of the albumin zone in agar electrophoresis, and

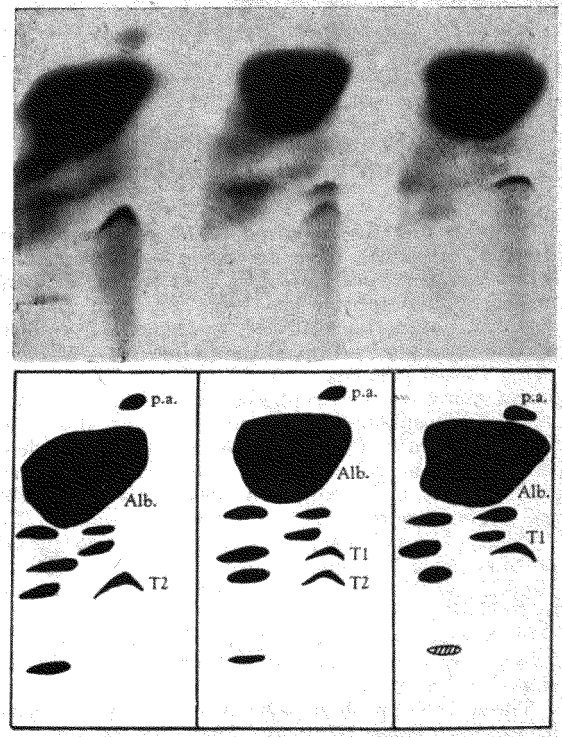


Fig. 1. The three thread-protein phenotypes in pig sera. The illustration shows the agar electrophoresis resolved albumin zones after further resolution in starch gel. The thread-protein phenotypes of the three serum samples are, from left to right, $T2$, $T1/T2$ and $T1$. In order to emphasize the thread proteins the starch gel was somewhat overloaded, resulting in poor resolution of the α-globulin zones. *p.a.*, Pre-albumin; *Alb.*, albumin; $T1$ and $T2$, thread proteins. Remaining zones are α-globulins. The β- and γ-globulins were not presented to the starch gel

well separated from the α-globulins in the second starch-gel electrophoresis. In two-dimensional electrophoresis the thread protein zones usually appear crescent-shaped, a phenomenon not shown by the remaining zones.

Preliminary mating data suggest that the three pig thread-protein phenotypes comprise the two homozygotes and heterozygote of a two-allele system. By analogy with the thread-protein system in cattle it is suggested that the two alleles be named pig T^A and T^B, so that the faster migrating zone $T1$ is seen in the presence of T^A and the slower zone $T2$ is seen in the presence of T^B. The three genotypes T^A/T^A, T^A/T^B and T^B/T^B would then be represented by phenotypes $T1$, $T1/T2$ and $T2$ respectively.

In common with all other mammals so far examined pigs also exhibit β-globulin polymorphism. Three β-globulin phenotypes have been seen in the samples examined, comprising the two homozygotes and heterozygote of a pair of β-globulin alleles, pig β^A and β^B. Each allele gives rise to three zones in starch gel in phosphate buffer, a rather faintly staining zone followed by a moderately staining zone and then by a more intensely staining zone. This is very similar to the situation found in sheep[2], goats[4] and mice (Ashton and Braden, unpublished work). In view of the increasing numbers of β-globulin alleles being found in other mammals, to date five in humans[5], ten in sheep (Ashton and Ferguson, unpublished work) and five in cattle[6], it is very likely that further examples will be found in pigs.

I wish to acknowledge the assistance of Dr. K. C. Sellers in procuring samples of pig sera.

G. C. ASHTON*

Animal Health Trust,
Farm Livestock Research Centre,
Stock, Essex.

* Present address: National Cattle Breeding Station, Commonwealth Scientific and Industrial Research Organization, Rockhampton, Queensland.

[1] Ashton, G. C., *Nature*, **182**, 65 (1958).
[2] Ashton, G. C., *J. Agric. Sci.* (in the press).
[3] Ashton, G. C., *Nature*, **182**, 1101 (1958).
[4] Ashton, G. C., and McDougall, E. I., *Nature*, **182**, 945 (1958).
[5] Giblett, E. R., Hickman, C. G., and Smithies, O., *Nature*, **183**, 1589 (1959).
[6] Ashton, G. C., *Nature*, **184**, 1135 (1959).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(27)

Reprinted from the
AUSTRALIAN JOURNAL OF BIOLOGICAL SCIENCES
VOLUME 14, NUMBER 2, PAGES 248–253, 1961

SERUM β-GLOBULIN POLYMORPHISM IN MICE

By G. C. Ashton and A. W. H. Braden

Reprinted for the
Commonwealth Scientific and Industrial Research Organization
Australia

SERUM β-GLOBULIN POLYMORPHISM IN MICE

By G. C. Ashton* and A. W. H. Braden†

[*Manuscript received November* 25, 1960]

Summary

Data are presented to show that there are three serum β-globulin types in laboratory mice controlled by a pair of alleles. Each allele appears to give rise to three electrophoretically distinct zones in starch gel. Within the inbred strain A/AGS there was variation between mice in the intensity of staining of the three zones. Reciprocal mating data gave no evidence of an effect of β-globulin type on segregation ratios as has been reported for cattle.

I. Introduction

Following the initial recognition of serum β-globulin polymorphism in cattle independently by Ashton (1957, 1958*c*), Hickman and Smithies (1957), and Smithies and Hickman (1958), it was soon established that the same phenomenon occurs in other mammals. Thus Smithies (1957) showed that human β-globulins are polymorphic while Ashton (1958*d*, 1958*e*, 1960*a*) and Ashton and McDougall (1958) demonstrated β-globulin polymorphisms in the serum of a number of farm animals including sheep, horses, goats, and pigs. Recently McDougall (personal communication) has found the phenomenon in red deer.

Other examples of serum protein polymorphism have been found. The original demonstration of qualitative inherited differences between the serum proteins of individuals was with the haemoglobin-binding α-globulins, termed haptoglobins (Smithies 1955). Polymorphic differences have also been found in the "thread-proteins" of cattle (Ashton 1958*a*) and pigs (Ashton 1960*a*), in the Sα proteins of cattle, and in the α-globulins and pre-albumins of horses (Ashton 1958*b*, 1958*d*). However, while all mammals so far examined have shown β-globulin polymorphism, the other polymorphisms have been seen in only one or a few species.

Relationships between β-globulin type and fertility (Ashton 1960*c*) and β-globulin type and economic factors (Ashton 1960*b*) have been examined in dairy cattle. It was found that matings between homozygous cows and homozygous bulls were significantly more fertile than matings between heterozygous cows and heterozygous bulls, a finding confirmed by Ogden (personal communication). Also it was shown that β-globulin type affected milk yield, β^{DD} cows being on average superior to β^{AA} cows by about 50 gal. These observations prompted a search for similar effects in other species. For this reason the serum β-globulins of another ruminant, the sheep,

* Division of Animal Genetics, C.S.I.R.O., Cattle Research Laboratory, Rockhampton, Qld.

† Division of Animal Physiology, C.S.I.R.O., Ian Clunies Ross Animal Research Laboratory, Prospect, N S.W.

are being investigated. It was also considered likely that the mechanisms underlying these β-globulin effects would be more easily identified in an animal more amenable to experimentation than the cow. Accordingly serum β-globulin polymorphism has been looked for, and found, in laboratory mice, guinea pigs, and rabbits. Laboratory mice have been studied in some detail, and the results are presented below.

The genetics of β-globulin polymorphism seems fairly simple and similar for each species. In each there appears to be a single locus controlling serum β-globulin type. So far, 5 alleles have been recognized in cattle (Ashton 1959*b*), 8 in the human (Giblett, Hickman, and Smithies 1959), 9 in sheep (Ashton and Ferguson, unpublished data), 2 in goats (Ashton and McDougall 1958), and 2 in pigs (Ashton 1960*a*). These results provided a baseline for the investigation of mouse β-globulin polymorphism.

II. Materials and Methods

(*a*) *Strains of Mice Examined*

Sera were examined from mice of the inbred strains C57BL/AGS, CBA/AGS, A/AGS, and 101/AGS. The number of animals tested from each strain was 27, 26, 27, and 4 respectively. In addition mice from three outbred stocks were examined: a randomly bred albino stock from the Department of Veterinary Physiology, University of Sydney, the Laboratory Animals Bureau's grey stock, and +T, $+t^3$, t^3t^3, and $+t^{12}$ mice derived from Tt^3 and Tt^{12} mutant lines maintained by Dr. S. Gluecksohn-Waelsch, New York. The number of mice examined from these three stocks were 26, 16, and 24 respectively. Three wild mice caught at Prospect were also tested.

Special mating groups were set up using mice of known serum β-globulin phenotype as indicated in Tables 1 and 2. Account was taken of the number of progeny born and the number weaned and, where possible, the phentoype of every mouse weaned was established. The mice were not tested until they had reached at least 5 weeks of age. Of those born 85% were weaned, and 78% were classified according to serum β-globulin type. Blood was obtained from the tail by the method described by Adams (1960), which enables samples of 0·5 ml to be taken rapidly; 20 mice could be bled in 30 min.

(*b*) *Starch-gel Electrophoresis*

Determination of the serum β-globulin phenotype was made by starch-gel electrophoresis using the horizontal technique developed by Smithies (1955) with minor modifications to the apparatus. The gels were prepared from hydrolysed starch (purchased from the Connaught Laboratories, University of Toronto, Canada) using phosphate buffer at pH 7·6 as described elsewhere (Ashton 1958*c*) for cattle serum proteins. Alternatively, we have used the discontinuous buffer system of Poulik (1957) as modified by Dr. K. A. Ferguson. With this system the electrolyte in the electrode compartments is a solution containing 1·2 g of lithium hydroxide and 11·8 g of boric acid per litre. The gel was prepared with a buffer made by adding 90 volumes of a solution containing 1·6 g citric acid and 6·2 g tris(hydroxymethyl)aminomethane per litre to 10 volumes of electrolyte. With this system and an applied voltage across the gel of about 10–12 V/cm the β-globulin zones were effectively separated in about 3 hr. The serum samples were inserted on rectangular pieces of filter paper

1 cm wide. To avoid irregularity in the final pattern the paper inserts were removed 15 min after the electrophoresis commenced. Several samples and a reference sample were run side-by-side on each gel. After electrophoresis the gels were slit lengthwise and the two exposed surfaces stained with nigrosine (Ashton 1958c). The β-globulin phenotype was assessed by comparison of the patterns given by the samples with that of the reference serum on the same gel.

The characterization of the zones separated from mouse serum by one-dimensional electrophoresis was aided by the two-dimensional electrophoresis technique of Smithies and Poulik (1956), first in agar in borate buffer (Ashton 1958c) and then in starch gel in phosphate buffer.

TABLE 1

SEGREGATION OF MOUSE SERUM β-GLOBULIN TYPES

Parental Phenotypes	No. of Offspring of Phenotype		
	β^{AA}	β^{AB}	β^{BB}
$\beta^{AA} \times \beta^{AA}$	*	—	—
$\beta^{AA} \times \beta^{AB}$	70	75	—
$\beta^{AA} \times \beta^{BB}$	—	91	—
$\beta^{BB} \times \beta^{BB}$	—	—	*
$\beta^{BB} \times \beta^{AB}$	—	167	172
$\beta^{AB} \times \beta^{AB}$	6	15	5

* Matings between homozygotes were not specifically examined, but numerous examinations from animals of several strains (inbred and outbred) revealed only one type within each strain.

III. RESULTS

(a) *Serum β-Globulin Types*

Plate 1, Figure 1, shows three β-globulin phenotypes which were found during this work. Phenotype β^{BB} occurred as the sole β-globulin type in the inbred strains C57BL, A, and 101, in three outbred stocks of different origin, and in the three wild mice tested, while phenotype β^{AA} was seen in only one strain (CBA), where it was the sole type. The third phenotype was produced by crossing animals of phenotypes β^{AA} and β^{BB}. The progeny were all of the third phenotype β^{AB}. These results, and the progeny totals from the various mating types shown in Table 1 make it clear that β-globulin polymorphism in the mice examined is controlled by two alleles which we have called β^{A} and β^{B}, so that genotypes β^{A}/β^{A}, β^{A}/β^{B}, and β^{B}/β^{B} are represented by phenotypes β^{AA}, β^{AB}, and β^{BB}. Further, each allele controls three protein zones as resolved by starch-gel electrophoresis: a fast-moving, rather faint zone not easily seen in all gels, an intermediate, more intensely staining zone, and a slow-moving, intensely staining zone. Although the point has not been checked the different degrees of staining probably represent corresponding variation of quantity of protein present in the three zones, rather than different binding capacities of the individual proteins for nigrosine.

Usually the relationship between the intensity of staining of constituent zones for any β-globulin allele within a given species remains reasonably constant. However, within the A/AGS inbred line we have seen two β^{BB} types (Plate 1, Figs. 2 and 3). Each gives three zones in starch gel, the mobilities of the three zones being identical for each

TABLE 2

SEGREGATION DATA FROM RECIPROCAL MATINGS

Parental Phenotypes ♀	Parental Phenotypes ♂	Mating Group No.	No. Litters	No. Born	No. Weaned	No. of Female Progeny: Homozygous	No. of Female Progeny: Heterozygous	No. of Male Progeny: Homozygous	No. of Male Progeny: Heterozygous
β^{AA}	β^{AB}	3	2	15	14	3	4	1	6
		4	2	8	8	1	3	4	0
		5	4	18	17	4	1	1	4
		6	3	12	9	1	2	2	4
		21	2	17	13	5	3	3	2
		22	2	12	7	1	3	3	0
Total			15	82	68	15	16	14	16
β^{AA}	β^{AA}	1	5	30	30	7	6	6	9
		10	3	34	25	6	4	10	5
		11	4	29	23	5	6	3	5
		12	3	26	25	4	7	7	6
Total			15	119	103	22	23	26	25
β^{BB}	β^{AB}	7	11	100	88	23	23	25	14
		8	9	77	67	14	19	13	15
		9	10	99	85	22	21	24	17
Total			30	276	240	59	63	62	46
β^{AB}	β^{BB}	2	4	33	27	9	10	4	4
		13	3	29	27	10	4	7	5
		14	4	31	30	9	5	5	5
		15	4	43	34	4	10	3	15
Total			15	136	118	32	29	19	29
Totals			75	613	529	128	131	121	116

"subtype". The most common type has zones which, in order of decreasing mobility, stain faintly, moderately, and intensely. The less common type has corresponding zones staining more or less evenly. When crossed with β^{AA} mice the resulting heterozygotes each showed three zones of corresponding mobility and were indistinguishable subjectively.

(*b*) *Reciprocal Mating Data*

Asymmetrical segregation ratios were obtained from matings between cattle of certain β-globulin genotypes (Ashton 1959*a*), so that there was a deficiency of offspring unlike the mother. This phenomenon was sought in mice.

Disturbed segregation ratios are most readily detected when two progeny phenotypes are expected in equal numbers from a mating type. Mice were therefore mated in four ways: $\beta^{AA}♀ \times \beta^{AB}♂$, and its reciprocal $\beta^{AB}♀ \times \beta^{AA}♂$; $\beta^{BB}♀ \times \beta^{AB}♂$, and its reciprocal $\beta^{AB}♀ \times \beta^{BB}♂$. The numbers of progeny observed of each phenotype is shown in Table 2. In none of the groups did the observed numbers differ significantly from the expected 1:1 ratio when tested by χ^2, nor was there any evidence of a significant excess of offspring of one sex in the combined data.

IV. Discussion

In common with other mammals the β-globulins of mice are polymorphic. As with all other examples of mammalian β-globulin polymorphism each allele appears to give rise to a group of zones on starch gel. In mice there seem to be three distinct zones per allele, in cattle four (Ashton 1959*b*), in sheep and goats two (Ashton and McDougall 1958), in horses three (Ashton 1958*e*), and in pigs three (Ashton 1960*a*). Recently Harris, Pennington, and Robson (1960) have demonstrated that there are probably two zones on starch gel for each β-globulin (transferrin) allele in the human.

It is possible that the β-globulin zones produced in each species from one allele are polymers of differing molecular size of a basic polypeptide. These would be separated in starch gel by the sieve-like action of the gel (Smithies 1955). However, the charge on each polymer would be the same, and supporting media not showing the sieve effect would not be expected to resolve the polymers. It has been demonstrated previously (Ashton and McDougall 1958) that while the β-globulin types in cattle, sheep, and goat sera may be recognized by paper electrophoresis, each allele gives rise on paper to one zone only, which cannot be resolved further.

The serum β-globulins of mice are unusual in that the relative staining intensities of the zones produced by an allele may differ between individuals within the same strain. Three individuals, showing the unusual pattern, from the A/AGS inbred line, were bled on two occasions about 2 weeks apart and the pattern had remained constant. The variation in relative staining intensity (and presumably in quantity of protein) of the zones in mice suggests a mechanism controlling the relationship between the constituent zones, although there is no evidence to distinguish between a genetic or physiological mechanism. The significance of the phenomenon is not known. However, it has been observed that in two sublines of the inbred strain, recognized by rejection of homografts, two individuals from one line had one β-globulin subtype, and two from the other line the other.

The data show no evidence of the disturbed segregation ratios that have been found in cattle, i.e. there is no consistent excess of female offspring of the same phenotype as the mother. It has been established that fertility in cattle is influenced by parental β-globulin type (Ashton 1960*c*), but that the mechanism is probably indepen-

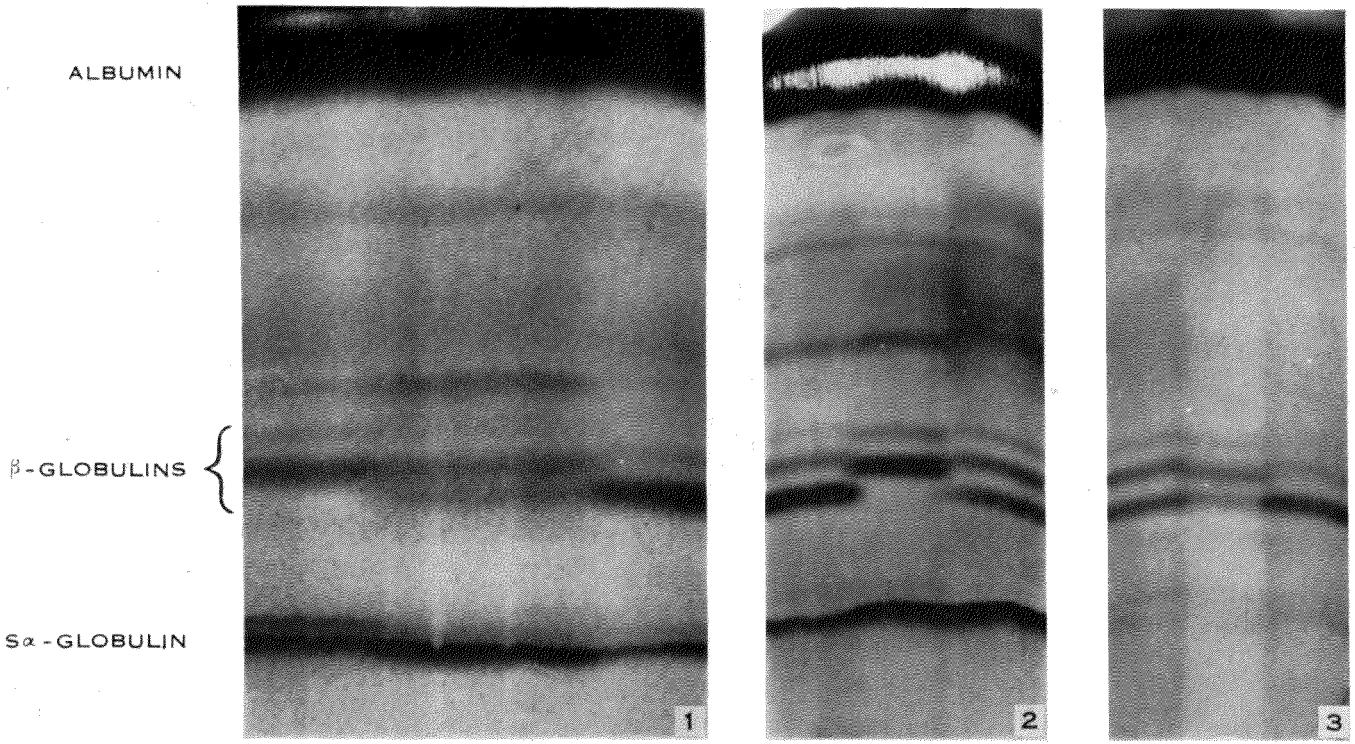


Fig. 1.—Anodic side of starch gel showing the stained protein zones from three different mouse sera after electrophoresis. The β-globulin phenotypes are, from left to right, β^{AA}, β^{AB}, and β^{BB}.

Fig. 2.—As for Plate 1, Figure 1, showing, from left to right, the phenotypes β^{BB}, β^{AA}, and modified β^{BB}. The latter phenotype illustrates clearly that each β-globulin allele produces three zones in starch gel.

Fig. 3.—As for Plate 1, Figures 1 and 2, showing, from left to right, the phenotypes β^{BB} modified, β^{AB}, and β^{BB}.

dent of that causing aberrant segregation ratios. The fact that the β-globulin mating groups in mice do not give asymmetrical segregation ratios does not necessarily mean therefore that parental β-globulin type has no effect on fertility.

V. Acknowledgments

The authors wish to acknowledge the capable technical assistance of Miss Jennifer Cullen and Mr. C. Bloomfield.

VI. References

Adams, K. M. (1960).—*J. Clin. Path.* **13**: 265.
Ashton, G. C. (1957).—*Nature* **180**: 917.
Ashton, G. C. (1958*a*).—*Nature* **182**: 65.
Ashton, G. C. (1958*b*).—*Nature* **182**: 193.
Ashton, G. C. (1958*c*).—*Nature* **182**: 370.
Ashton, G. C. (1958*d*).—*Nature* **182**: 1029.
Ashton, G. C. (1958*e*).—*Nature* **182**: 1101.
Ashton, G. C. (1959*a*).—*Nature* **183**: 404.
Ashton, G. C. (1959*b*).—*Nature* **184**: 1135.
Ashton, G. C. (1960*a*).—*Nature* **186**: 991.
Ashton, G. C. (1960*b*).—*J. Agric. Sci.* **54**: 321.
Ashton, G. C. (1960*c*).—*J. Reprod. Fert.* **2**: (in press).
Ashton, G. C., and McDougall, E. I. (1958).—*Nature* **182**: 945.
Giblett, E. R., Hickman, C. G., and Smithies, O. (1959).—*Nature* **183**: 1589.
Harris, H., Pennington, D. G., and Robson, E. B. (1960).—*Biochem. J.* **74**: 44P.
Hickman, C. G., and Smithies, O. (1957).—*Proc. Genet. Soc. Canada* **2**: 39.
Poulik, M. D. (1957).—*Nature* **180**: 147.
Smithies, O. (1955).—*Biochem. J.* **61**: 629.
Smithies, O. (1957).—*Nature* **180**: 1482.
Smithies, O., and Hickman, C. G. (1958).—*Genetics* **43**: 374.
Smithies, O., and Poulik, M. D. (1956).—*Nature* **177**: 1033.

SERUM TRANSFERRINS IN SOME AFRICAN ANTELOPES

by G. C. ASHTON[1] and W. R. CARR[2]

SUMMARY

Sera from fifty-seven eland from game parks in East Africa and Southern Rhodesia were examined by starch gel electrophoresis. Twenty-six different transferrin phenotypes were observed. The appearance of the phenotypes and limited mating data supported the hypothesis that this polymorphism is controlled by nine autosomal transferrin alleles. Sera from thirty-one Grant's gazelles showed three transferrin phenotypes as did sera from thirty-one Thomson's gazelle. Transferrin polymorphism was not found in sera from seventy wildebeest, or thirty-one waterbuck.

INTRODUCTION

Transferrin polymorphism in cattle was first described by Ashton (1958), and Smithies and Hickman (1958). It has also been found in many other species including humans, chimpanzees, monkeys, sheep, goats, deer, reindeer, pigs, horses, fowl and mice (see Ashton and Ferguson (1963) for references). There are nevertheless species in which transferrin polymorphism has been sought without success. It is not yet clear why this distinction exists, but when more species have been examined a pattern may emerge which will increase understanding of the biological significance of transferrin polymorphism.

This communication reports studies on the transferrins of five different species of antelope. Most of the animals were from East Africa, but results include data from sera obtained from Southern Rhodesian eland.

MATERIALS AND METHODS

Serum samples from eland, Grant's gazelle, Thomson's gazelle, wildebeest (gnu) and waterbuck were made available by Mr. W. Plowright of the East African Veterinary Research Organization. Serum samples from eland in Southern Rhodesia were supplied by the Department of National Parks and Wildlife Management. The sera were obtained from:—

1. Eland (*Taurotragus oryx*). (*a*) 28 eland shot in the Ngorongoro/Serengeti area of Northern Tanganyika, (*b*) 17 animals shot in Southern Rhodesia game parks, and (*c*) 12 animals in an experimental herd of semi-domesticated eland of the Southern Rhodesia Department of National Parks and Wildlife Management.
2. Grant's Gazelle (*Gazella granti*). 31 animals shot in the Ngorongoro/Serengeti area of Northern Tanganyika.
3. Thomson's Gazelle (*Gazella thomsoni*). 31 animals, some of which were shot at Naivasha (Kenya) and the remainder in the Ngorongoro/Serengeti area.
4. Blue, or white bearded wildebeest (*Gorgon taurinus*). 54 wildebeest from the Ngorongoro/Serengeti area and 16 from Kajaido.
5. Waterbuck (*Kobus defassa*). 31 waterbuck shot in the Queen Elizabeth National Park, Uganda.

Blood samples were collected into sterile bottles and, where possible, separated within 24 hours. They were then transferred to deep-freeze at −20/25°C or stored on ice until transferred to deep-freeze. Some of the Southern Rhodesian eland sera were treated with merthiolate prior to freezing.

Starch gel electrophoresis was carried out in a discontinuous boric acid/lithium hydroxide, tris/citric acid system (Ashton and Lampkin, 1964). Sera from the East African and Rhodesian eland were compared directly on the same gels. Transferrin zones were identified on the starch gels by autoradiographic examination after treatment with iron-59 and also by staining with nitroso-R salt.

RESULTS

Transferrin polymorphism was found in three of the five species of antelope examined.

ELAND

A complex transferrin polymorphism was found in both the East African and Southern Rhodesian samples. Twenty-six phenotypes were observed. The interpretation of these was aided by the observation (Ashton and McDougall, 1958) that in other species a transferrin allele usually gives rise to a group of zones of characteristic appearance after staining, and further that the different alleles give analagous groups of zones but of differing electrophoretic mobility. In the case of the eland sera the group of zones appeared

[1] Cattle Research Laboratory, Commonwealth Scientific and Industrial Research Organization, Rockhampton, Queensland, Australia. Present address: Department of Genetics, University of Hawaii, Honolulu 14, Hawaii.

[2] Animal Productivity Research Team, Agricultural Research Council of Central Africa, Chilanga, Zambia.

as three zones staining very faintly, moderately, and intensely in decreasing order of mobility (Plate 1). The nine presumed alleles, based upon the mobility of these zone groups, were coded in decreasing order of mobility; eland Tf^A, Tf^B, . . . Tf^H, Tf^J. The relative mobility of the zones is shown diagrammatically in Figure 1.

The distribution of phenotypes was as follows: (*a*) East African sera: one each of Tf A/A, A/D, A/F, A/G, B/B, B/C, B/D, B/F, F/H, C/F, C/H, D/D, D/J, E/F, E/J, and F/H, two each of C/G, D/H, and F/G, and three each of D/E and D/F. (*b*) Southern Rhodesian sera: one each of Tf A/D, D/F and G/H, two each of A/B, A/H, B/F, B/H, E/G, F/F and F/G, three of A/G, four of E/F and five of A/F.

Eland Tf^C and Tf^J were not represented in the Southern Rhodesian samples, but the remaining seven alleles were represented in samples from both areas.

Limited breeding data were obtained from an experimental herd of semi-domesticated eland in Southern Rhodesia. Serum samples from the sire(s) were not available, but it was thought that only one sire was responsible for the offspring sampled. Dam Tf E/F gave progeny Tf B/F, E/G, and E/F, Dam Tf A/H gave progeny Tf A/G and G/H, and dam Tf A/B gave progeny Tf A/F. Assuming that only one sire was involved and that his genotype was Tf F/G there is one discrepancy (progeny B/F) from the multi-allelic hypothesis proposed.

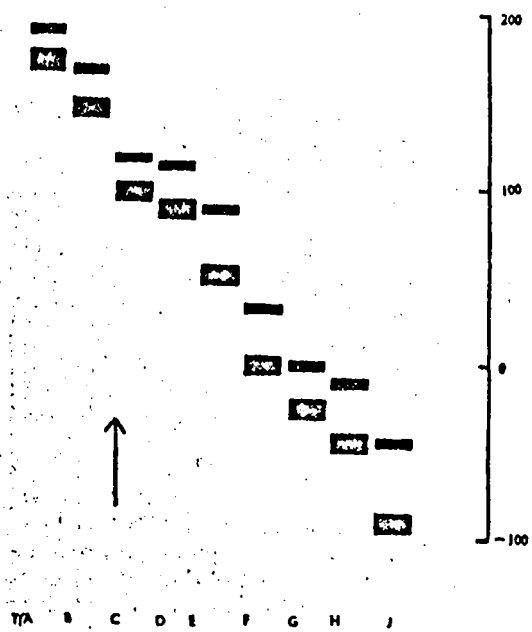


FIGURE 1

Relative mobility of the zones produced by the nine eland transferrin alleles. The distance between the slowest zones of those produced by Tf^C and Tf^F was arbitrarily designated as 100 units, and the mobilites of the other zones is expressed in these units.

Thomson's Gazelle

Sera from thirty-one animals showed three phenotypes. Twenty-five of these were alike and showed a group of three iron-binding zones. In descending order of mobility, these stained moderately, intensely, and intensely. A further serum showed a similar group of three zones with a slower mobility. Four sera showed both groups of three zones (Plate 1). It seems probable that these phenotypes represented the two homozygotes and the heterozygote of two transferrin alleles, Thomson's gazelle Tf^A and Tf^B, the zones produced by Tf^A being faster. No mating data were available to check this hypothesis.

Grant's Gazelle

Sera from thirty-one animals were examined. Twenty-four of these were the same and showed a group of three zones staining as in the previous species, i.e. moderately, intensely, and intensely in decreasing order of mobility. Four of the sera showed a similar group of three zones of somewhat slower mobility, while the final three showed zones indistinguishable from those produced by a mixture of the other two phenotypes (Plate 1). It was concluded that the animals examined exhibited transferrin polymorphism similar to that shown by Thomson's gazelle, i.e. a two allele system, Grant's gazelle Tf^A and Tf^B. However the zones in each species differ in mobility, and must therefore be the products of different genes.

Wildebeest

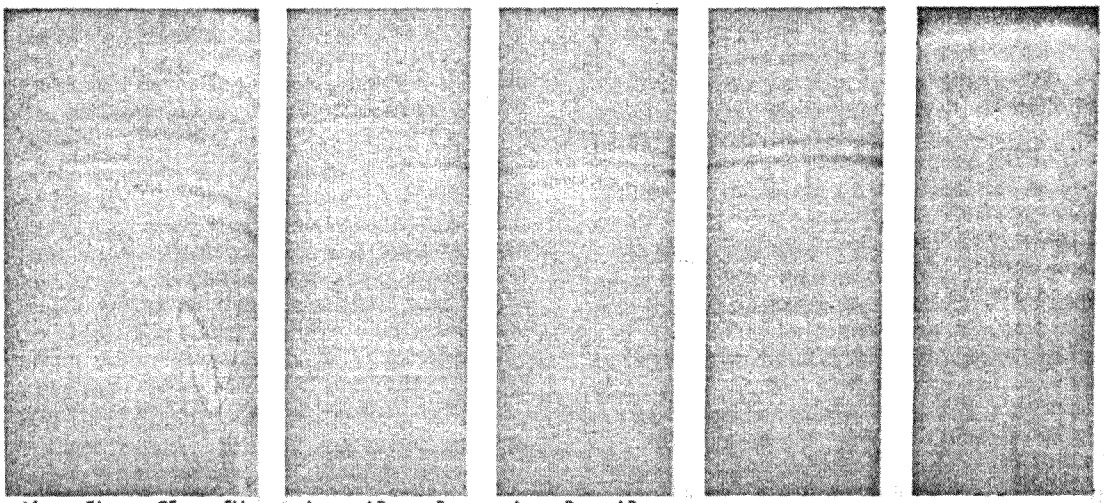
The transferrins from 70 wildebeest appeared as one phenotype, and consisted of a group of three zones staining faintly, moderately, and intensely in order of decreasing mobility (Plate 1). The observation of one phenotype only was interesting because the animals from the two areas sampled were apparently zoologically dissimilar.

Waterbuck

All sera showed the same transferrin pattern, consisting of a group of three zones. The zones were relatively widely spaced compared with those of the other antelopes examined and all stained with approximately equal intensity (Plate 1).

PLATE I

Examples of transferrin polymorphism in some African antelopes. The eland gel carries four different serum samples, the other gels each have three samples.



DISCUSSION

The complexity of eland transferrin polymorphism was an unexpected finding. The possibility that part of this complexity was artefactual in origin was unlikely in view of the absence of any spurious patterns, such as those observed in sheep sera when bacterial neuraminidase has acted on the sialic acid of the transferrin molecules (Ashton and Ferguson, 1963). Additionally, comparison of merthiolate treated samples from Southern Rhodesia with non-preserved samples stored for two years showed no evidence of change in the transferrin region in the latter samples, although the mobility of their albumin zones altered slightly.

The evidence for the existence of a complex transferrin polymorphism in eland is strengthened by the replication of phenotypes found in animals from widely scattered areas and by the limited mating data available. Nine alleles were represented in the 28 Uganda samples, and in the Southern Rhodesian samples seven of these alleles were represented in the 17 sera from the game park eland, and six of these alleles in nine sera from the experimental herd.

These results with five species of antelope illustrate the wide range of transferrin phenotypes found within a few closely related genera. Some species such as eland, sheep, cattle and humans show multi-allelic transferrin polymorphism. Others, such as wildebeest and waterbuck have not yet been found to be polymorphic for transferrins.

It was established by Fisher (1922) that, in an infinite population, heterozygote superiority in fitness for a pair of alleles leads to a stable polymorphism. With more than two alleles the maintenance of a stable equilibrium is more complex. The requisite conditions were considered by Kimura (1956) who stated that the complexity of these conditions does not change the general conclusion that over-dominance is an effective way of maintaining a multi-allelic polymorphism in a population.

Kimura and Crow (1964) investigated the number of alleles which can be maintained in a finite population. For a locus at which two or more alleles are maintained by selective superiority of the heterozygotes, the average fitness of the population is increased with a larger number of alleles. This is counter-balanced by the effect of random drift in reducing the number of alleles, which increases greatly as the number of alleles increases. With a population of a certain size and mutation rate there will be a maximum number of alleles maintainable for a given level of heterozygote advantage. This will correspond to the minimum segregation load.

Kimura and Crow (1964) show these relationships graphically. For nine alleles and an arbitrarily chosen mutation rate of 10^{-5} the effective breeding population size would have to be approximately 300,000 if the alleles were selectively neutral, 55,000 if the selective disadvantage of the homozygotes were 0·1%, and 15,000 for 1%. The effective breeding population of eland in Africa at the present time is unlikely to be 300,000 but is probably more than 55,000, although no figures are available. This could indicate mutually heterotic selective advantage for the alleles persisting, but the extent of this need not be very great.

However, this type of inference presents some major difficulties. Perhaps the main one lies in the fact that eland herds are considered by some authorities in the territorial game departments to be largely closed and it therefore means that the eland populations of East, Central and Southern Africa cannot be regarded as a single population. The different groups or herds live in widely different environments and this leads to further conjecture on the role of adaptive polymorphism.

Applying Kimura and Crow's projections in another way two transferrin alleles could be maintained in an effective breeding population of about 25,000 even if selectively neutral, at a mutation rate of 10^{-5}. If, as it appears likely, the effective population number for wildebeest is as high as 25,000 and there is no selection advantage, then it is surprising that wildebeest show no transferrin polymorphism.

There seems little doubt that in antelopes at least the selective advantage of transferrin polymorphism is not high.

ACKNOWLEDGEMENTS

We are grateful to Mr. W. Plowright of the East African Veterinary Research Organization, Kenya, and Dr. H. H. Roth of the Department of National Parks and Wild Life Management, Southern Rhodesia, for providing the serum samples examined, and to Mr. P. M. Burrows, A.R.C. Biometrics Team for advice. One of us (G. C. A.) thanks the Director of the East African Veterinary Research Organization, Muguga, Kenya, for providing laboratory facilities while working as a guest of the Organization.

REFERENCES

ASHTON, G. C. (1958). Genetics of β-globulin polymorphism in British cattle. *Nature, Lond.*, **182**, 370-372.

ASHTON, G. C. and FERGUSON, K. A. (1963). Serum transferrins in Merino sheep. *Genet. Res.*, **4**, 240-247.

ASHTON, G. C. and LAMPKIN, G. H. (1964). Serum albumin and transferrin polymorphism in East African cattle. *Nature, Lond.*, in press.

ASHTON, G. C. and MCDOUGALL, E. I. (1958). β-globulin polymorphism in cattle, sheep and goats. *Nature, Lond.* **182**, 945-946.

FISHER, R. A. (1922). On the dominance rates. *Proc. Roy. Soc. Edin.* **42**, 321-341.

KIMURA, M. (1956). Rules for testing stability of a selective polymorphism. *Nat. Acad. Sci. Proc* **42**: 336-340.

KIMURA, M. and CROW, J. F. (1964). The number of alleles that can be maintained in a finite population. *Genetics* **49**, 725-738.

Amer. J. Hum. Genet. (in press)

(29)

C5 TYPES OF SERUM CHOLINESTERASE IN A BRAZILIAN POPULATION

By

G. C. Ashton
Department of Genetics
University of Hawaii
Honolulu, Hawaii 96822

and

N. E. Simpson*
Departments of Paediatrics
and Biology
Queen's University
Kingston, Ontario

* Queen Elizabeth II Scientist

Supported in part by U. S. Public Health Service grant GM 10424 with the collaboration of the University of Sao Paulo and the Departamento de Imigracao e Colonizacao, Sao Paulo, in part by U. S. Public Health Service grant 1 R01831-01 and in part by a Medical Research Council of Canada grant MA 1876.

During the year beginning June, 1962, N. E. Morton and his colleagues collected blood samples from 1068 families from northeastern Brazil (Morton, 1964). The primary purpose of the study was to determine the contributions of antigenic incompatibility, heterozygote advantage, meiotic drive and rare recessive genes to genetic load expressed as fetal and post-natal deaths. The samples were analyzed for a variety of blood groups and for several protein and enzyme polymorphisms. The data presented here relate to the C5 serum cholinesterase variants which are detectable by starch gel electrophoresis as first described by Harris, Hopkinson and Robson, (1962).

The C5 cholinesterase system was so called by Harris, et al. (1962), because of the presence or absence of a protein in human serum with cholinesterase-staining activity, this C5 protein being accompanied by four other proteins (C1, C2, C3 and C4) with similar activity but faster mobility in starch gel electrophoresis at pH 6.0. The family data collected by Harris and his colleagues led them to suggest that individuals whose sera showed the C5 protein (C5+) were homozygous or heterozygous for an autosomal gene which determines the C5 protein. It has been suggested by some workers in the field that the gene for the C5+ protein be known as E_2^+ and its allele as E_2^- (Motulsky, 1964). However, occasional samples were tested in which the C5 zone was apparently absent, although family data suggested that these individuals were heterozygotes if the theory advanced was correct. Harris, Hopkinson, Robson and Whittaker (1963) pointed out that some heterozygotes either did not exhibit the C5 component, or else it was not detectable by their procedure and suggested that the genetic mechanism may be more complex. Subsequently, Harris, Robson, Glen-Bott and Thornton (1963) published evidence that the pair of genes controlling the C5 variants are at a different locus to the genes controlling

qualitative cholinesterase variation (E_1^u and E_1^a). Three phenotypes produced by the E_1 locus, usual, intermediate and atypical, are distinguished by dibucaine inhibition (DN) under certain standard conditions (Kalow and Genest, 1957). There are two other alleles at the E_1 locus: E_1^f (Harris and Whittaker, 1962) and E_1^s (Simpson and Kalow, 1964), both of which are rare. Although the main interest in the present report is in the C5 cholinesterase system, E_1 phenotypes were also determined on these sera.

MATERIAL

The investigation of the Brazilian material for C5 type was carried out in two parts. In Toronto serum samples from 108 families were examined. These families were originally selected from the 1068 families by an agar-diffusion test (Harris and Robson, 1963), used as a screening test for sera containing atypical cholinesterase. These samples were subsequently tested by spectrophotometric methods for E_1 phenotype (Simpson and Kalow, 1965). Most of the samples (621) which had been selected by the agar diffusion method were also typed for C5 variants. In Honolulu all the parental sera of the Brazilian population were examined for C5 variants. All parental samples were run in duplicate, the samples and duplicates being run "blind" at random. If there was disagreement in classification of duplicates, a third "blind" test was done at random and the final classification was made using the two results which were in agreement. All filial sera were tested when one or both parents had C5+ sera, and filial serum samples from 297 of the 899 families in which both parents were negative were tested.

In addition, sera from 163 randomly selected healthy Canadian individuals and from 317 Negroes (mainly male) who were blood donors in Seattle,

were typed for C5 in duplicate as described below, in Toronto. The Negro sera were kindly provided by Dr. E. R. Giblett, King County Central Blood Bank, Inc., Seattle.

METHODS

In Honolulu the gels were run in a horizontal apparatus (Ashton, 1965) for 16 hours at 4° C. In this system the electrolyte contained 39.5 g. citric acid and 11.1 g. of anhydrous lithium hydroxide per litre at pH 5.3. The gels were prepared in a pH 5.3 buffer solution made from 10 volumes of electrolyte and 90 volumes of a solution containing 5.5 g. of tris (hydroxymethyl) amino-methane and 4.2 g. of succinic acid per litre. An applied voltage of 150 volts across the apparatus gave a current of about 4 to 4.5 ma. per cm. width of 3 mm. thick gel. In both laboratories the hydrolyzed starch produced by Connaught Laboratories, Toronto, Canada, was used. Enzyme activity was detected by incubating the gels at 37° C. in a freshly prepared mixture of two solutions A and B (Solution A: 20-30 mg. of α-naphthyl acetate, 10 ml. ethanol or acetone, 90 ml. 0.9% saline. Solution B: 20-30 mg. Fast blue RR salt, 10 ml. 0.1 N. hydrochloric acid, 5 ml. 1% calcium chloride, 85 ml. 0.025 M pH 8.9 borate buffer). The cholinesterase zones usually appeared after about 30 minutes incubation. (Fig. 1).

Starch gel electrophoresis was carried out in Toronto in Smithies (1959) vertical apparatus using a pH 5.3 discontinuous buffer system described by Harris, Robson, Glen-Bott and Thornton (1963); the concentration of starch was about 14%. Enzyme activity was detected as in Honolulu substituting Naphthanil Diazo blue for Fast Blue RR salt. The gels were run for 17 hours at 100 volts and 25 ma. at about 15° C. Harris, Hopkinson, Robson and Whittaker (1963) had

noted that unidimensional electrophoresis was more sensitive, although separation not as good, for detecting the C5 zone than their two-dimensional filter paper/starch gel procedure. The latter technique, however, was carried out in Toronto on a few samples chosen at random to confirm that the C5 zone corresponded with that seen by Harris and his colleagues.

RESULTS

Repeatability of Phenotyping

Harris, Hopkinson, Robson and Whittaker (1963) noted difficulty in typing some sera, and used the notation C_5 + (?) to indicate phenotypes doubtfully positive. These were individuals in which the C5 zone was apparently present in unidimensional starch gel electrophoresis, but could not be detected in two-dimensional electrophoresis. In some cases, pedigree data suggested that such individuals were carrying the C5+ gene. Harris, Robson, Glen-Bott and Thornton (1963) estimated that the undetected carriers of the C5+ gene occurred about one in fifty in a randomly selected English population.

Similar difficulties were noted in the present study. The repeatability of phenotyping for the first 1,010 replicates of sera tested in Honolulu was less than that experienced with other protein and enzyme polymorphisms (Table 1). About 95% of the individuals were apparently typed correctly the first time. About 5% of the initial typings did not agree with the second typing. After the third typing of these samples it was found that about four-fifths of them typed once as positive or query positive were subsequently judged to be negative. One-fifth typed once as negative were subsequently judged to be positive. The discordance between duplicate typings decreased with the second thousand samples. Nine positive or query positives were judged negative on the second and third runs, and no negatives becoming positive on the second run were found.

A number of sera were examined independently in Toronto and Honolulu. Among 193 parental sera there were no discrepancies. Among 113 filial sera tested at both locations two samples, from children of C5- parents, were judged C5- in Honolulu and C5+ in Toronto. Both children were in the same family. On repetition at a later date in Toronto these two sera were still judged positive. Extensive studies of blood group and serum protein markers from Dr. Morton's data did not suggest illegitimacy in this family.

Frequency of C5 Phenotypes

There were 1053 serum samples from male parents available for typing, and of these 98 (9.3%) were C5+. There were 1049 serum samples available from female parents, of which 72 (6.9%) were C5+. The difference between the male and female parents in the proportion of C5+ phenotypes is significant ($P < 0.05$).

Comparison with the frequencies of C5+ phenotypes in other populations (Table 2) shows that there is no difference between them except that the frequency of C5+ phenotypes in the Tristan da Cunha Islanders (Harris, Hopkinson, Robson and Whittaker, 1963) is higher than in the other populations. The number of C5+ phenotypes in the British, Canadian and Negro populations was small, and at the time these were tested the possible sex difference for C5+ phenotypes among adults was not appreciated. The majority of the British and Negro populations consisted of males.

Independence of the Two Cholinesterase Loci

Table 3 shows the distribution of C5 phenotypes within phenotypes at the cholinesterase E_1 locus in 2,102 parental sera. The usual phenotype (U) was determined either by a negative result from the agar diffusion test, by the spectrophotometric method or both, and all other phenotypes by the spectro-

photometric method (see Simpson and Kalow, 1965). The proportion of C5+ phenotypes is similar for both the usual and intermediate phenotypes, and there is no evidence of inter-relationship between the phenotypes produced by the two loci. Harris, Robson, Glen-Bott and Thornton (1963) and Simpson (1966) have presented data from double backcross matings showing that the two loci are not closely linked.

A Further Electrophoretic Cholinesterase Variant

One serum sample from the father of family No. 192 showed an unusual zone after electrophoresis, located between the origin and the C4 cholinesterase zone (Fig. 1). This zone has been tentatively coded C6. A further zone, very much fainter, was also present in this serum, migrating between the origin and C6. Sera from all members of the family, i.e., C6 positive father, mother, and the four children tested, were C5 negative. Only the father showed the C6 zone. The cholinesterase activity of his serum was 171 units, just below the average (188) for the UC5- phenotype (Simpson, 1966). The dibucaine numbers of this family ranged from 82 to 87, and the fluoride numbers from 62 to 67 which are typical of UC5- phenotype.

Segregation from C5 Matings

Table 4 shows the segregation of progeny from the four possible matings between C5 phenotypes. Although 1053 male parent samples and 1049 female parent samples were typed, only 1038 families with both parents typed are considered in Table 4. There were 884 families with both parents typed as C5-, but 1132 children of only 297 of these families were examined for C5 type. All sera from children whose parents both had negative sera were negative, with the exception of two children from one family of eight children noted above whose sera were typed as positive in Toronto but not in Honolulu. With these

two exceptions the phenotypes of the offspring produced from the four mating combinations were in accord with Harris' hypothesis of genetic control by two autosomal alleles. However, the distribution of offspring phenotypes observed for the three matings with at least one parent whose serum was C5+ differed significantly ($P < 0.01$) from that expected from the calculated parental gene frequencies (Table 4). In the calculation of expected phenotype distributions both mean gene frequencies calculated from all parents irrespective of sex, and individual male and female gene frequencies were used. There was little difference in the expected numbers of each type of progeny by the two methods of calculation. From the three segregating matings there was a significant lack of positive children and a corresponding excess of negative children compared with expectation.

Fertility of C5 Matings

Table 5 shows the number of live children, fetal deaths and post-natal deaths for each mating. (We are indebted to Dr. N. E. Morton for allowing us to extract this information from his Brazilian data). There are no significant differences between matings in any of these three parameters.

Age Distribution of Phenotypes

The mean ages of the typed children from the various matings are shown in Table 6. Data of these sort which are not normally distributed are most easily interpreted by comparing phenotypic frequencies within age groups. Table 6 shows the distribution of C5 positive and negative male and female children from each mating in the age groups 0-13 years and 14 years and over. Very similar results are obtained for distributions 0-12, 13 and over, and 0-11, 12 and over. The relative proportions of children in each age group were

compared between sex and phenotypic classes by χ^2 analysis. There are no differences in the proportions of C5 negative male (0.27), positive female (0.24), and negative female (0.29) children between the two age groups in the pooled data. However, the proportion of C5+ male children (0.14) in the older age group differs significantly from the proportion of C5- males ($P < 0.01$), C5- females ($P < 0.01$), and C5+ females ($P < 0.05$). There were relatively fewer C5+ males 14 years and over. This was a consistent finding for each of the segregating matings. It implies a relative deficiency of C5 positive males at or after adolescence compared with females. This is not consistent with the observation of an excess of C5+ male parents. The mean ages of the C5 positive and negative parents did not differ significantly for each sex, viz., C5+ males 42.97 years, C5- males 43.24 years; C5+ females 36.21 years, C5- females 36.36 years.

DISCUSSION

The data show three major significant effects: more C5+ male than female parents, a deviation from expectancy in the segregation ratios, and fewer C5+ males among those children 14 years and over. We have considered various models by which these results may be rendered consistent. Among these were alternative modes of inheritance, the possibility of selection, and typing inconsistencies. The latter seems to explain the data in the least contentious manner, but none of the models has proved completely satisfactory.

Alternative Genetic Models

Harris, Hopkinson, Robson & Whittaker (1963) proposed that individuals whose sera showed the C5 protein are homozygous or heterozygous for an autosomal gene which produces this protein, and individuals lacking the protein are

homozygous for an alternative allele. However, these authors were careful to point out that some parents who were presumed to be C5- from pedigree data gave rise to positive children, and advised caution in the acceptance of their hypothesis. In the data presented here out of 297 families with C5- parents there was one which had two apparently positive children. These children (one male, one female) were consistently classified as positive in Toronto in blind trials, but were classified as negative in Honolulu.

With this exception the types of offspring produced from the possible matings in our family data support the hypothesis that the C5+ phenotype is usually a heterozygote for the gene producing the C5 protein, but the segregation data suggest that other factors are involved. Alternative genetic models, including sex-linkage and a sex-linked modifier have been considered, but have not given a good fit to the segregation data.

Selection

There is a significant excess of C5- and a corresponding lack of C5+ children from the three matings showing segregation (Table 4). This might imply strong selection against positive children, assuming the genetic model is correct. However, there is no evidence of such a strong effect in the fertility data (Table 5).

Another effect which might suggest selection is the significantly lower proportion of C5+ boys in the 14 years and older age group compared with boys in the under 14 years age group. This effect is not shown by the female children, and an excess of C5+ mothers compared with C5+ fathers would be anticipated. The converse is true however, there being significantly more C5+ fathers than mothers.

It seems that the data do not show significant or consistent evidence of selection.

Phenotyping

Cholinesterase C5 typing is more subjective than some other protein or enzyme typings. At least in the early stages of experience with this technique there seems to be poorer reproducibility than is customary with starch gel phenotyping. Increasing familiarity with the system improves the reproducibility of replicate typings. The good but not perfect agreement between independent typings in Toronto and Honolulu (two discrepancies out of 306 samples tested) emphasizes the difficulties of assigning a phenotype to some serum samples. Harris, Hopkinson, Robson and Whittaker (1963) recognized this problem, and assigned the phenotype C5+? to some samples which they could not classify as positive in two-dimensional gels but which did not appear to lack the C5 component completely in one dimensional gels.

There seems to be a tendency to classify negatives as positive, judging by the Honolulu repeatability data. If some of the sera typed as positive were in fact genetically negative our data could be explained. Thus the predominance of negative children from matings with one or both parents typed as positive is understandable if some of these parents were really negative. Similarly the significant differences in frequency of C5+ fathers and mothers and in the age distribution in male progeny might be ascribed to mistyping rather than to a true effect.

The reasons for possible mistyping are not clear. The generally good agreement between Honolulu and Toronto suggests that an error in interpretation of a stained gel pattern is not the cause. One possibility is that physiological factors may cause an increase in concentration of an esterase component with similar mobility to C5, normally not detected by the staining procedure used. Another possibility is that serum storage may cause the production of a C5-like component. Harris, Hopkinson, Robson and Whittaker (1963) described "storage bands" S1 and S2, but state that at pH 6.0 S1 and S2 have a similar

mobility to C4. We do not think that we are typing S1 and S2 as C5 but other types of storage change cannot be excluded. However, if such changes had occurred they would reasonably be expected to be at random with respect to the samples collected, and would probably not result in significant and in some cases consistent differences of the type described.

SUMMARY

Cholinesterase C5 phenotypes of over one thousand Brazilian families were determined by starch gel electrophoresis. The proportion of C5+ phenotypes among male parents (9.3%) and female parents (6.9%) differed significantly ($P < 0.05$). Segregation of progeny from the four possible matings between C5 phenotypes differed significantly ($P < 0.01$) from expectation, more C5- children being found than expected. There was also a significant difference ($P < 0.01$) in the age distribution of the children's phenotypes; 29% of C5- female children, 27% of C5- male children, and 24% of C5+ female children were aged 14 years or older, but only 14% of C5+ male children were in this age range.

The reason for these significant effects is not known, but inconsistent phenotypic expression of the genotype seems the most plausible explanation at this time.

ACKNOWLEDGMENT

We wish to thank Dr. N. E. Morton for allowing us to examine the Brazilian serum samples and Mr. M. N. Dennis for technical assistance.

REFERENCES

ASHTON, G. C. 1965. Serum transferrin D alleles in Australian cattle. Aust. J. Biol. Sci. 18: 665-670.

HARRIS, H., HOPKINSON, D. A., and ROBSON, E. B. 1962. Two dimensional electrophoresis of pseudocholinesterase components in normal human serum. Nature (Lond.) 196: 1296-1298.

HARRIS, H., HOPKINSON, D. A., ROBSON, E. B., and WHITTAKER, M. 1963. Genetical studies on a new variant of serum cholinesterase detected by electrophoresis. Ann. Hum. Genet. 26: 359-382.

HARRIS, H., and ROBSON, E. B. 1963. Screening tests for the "atypical" and "intermediate" serum-cholinesterase types. Lancet II: 218-221.

HARRIS, H., ROBSON, E. B., GLEN-BOTT, A. M., and THORNTON, J. A. 1963. Evidence for non-allelism between genes affecting human serum cholinesterase. Nature (Lond.) 200: 1185-1187.

KALOW, W., and GENEST, K. 1957. A method for the detection of atypical forms of human serum cholinesterase. Determination of dibucaine numbers. Canad. J. Biochem. Physiol. 35: 339-346.

MORTON, N. E. 1964. Genetic studies of northeastern Brazil. Cold Spring Harbor Symp. Quant. Biol. 29: 69-79.

MOTULSKY, A.

SIMPSON, N. E. 1966. Genetic and non-genetic factors influencing cholinesterase activity in a Brazilian population. Amer. J. Hum. Genet.

SIMPSON, N. E., and KALOW, W. 1964. The "silent" gene for serum cholinesterase. Amer. J. Hum. Genet. 16: 180-188.

SIMPSON, N. E., and KALOW, W. 1965. Comparisons of two methods for typing of serum cholinesterase and prevalence of its variants in a Brazilian population. Amer. J. Hum. Genet. 17: 156-162.

SMITHIES, O. 1959. An improved procedure for starch-gel electrophoresis: Further variations in the serum of normal individuals. Biochem. J. 71: 585-587.

TABLE 1

AGREEMENT BETWEEN REPLICATES FOR C5 CHOLINESTERASE PHENOTYPING OF THE FIRST 1010 SAMPLES EXAMINED (HONOLULU DATA).

Run No. & phenotype recorded			No. of samples	Percent of total
1.	2.	3.		
C5-	C5-	...	889	94.8
C5+	C5+	...	68	
C5+ or C5+?	C5-	C5-	18	4.2
C5-	C5+ or C5+?	C5-	24	
C5-	C5+	C5+	3	1.0
C5+ or C5+?	C5-	C5+	8	

TABLE 2

COMPARISON OF FREQUENCIES OF C5+ PHENOTYPES IN DIFFERENT POPULATIONS.

Population	Phenotype C5+ No.	C5+ %	C5- No.	C5- %	Total
Brazilian parents	170	8.1	1932	91.9	2102
British*	13	5.2	235	94.8	248
Tristan da Cunha*	36	16.9	177	83.1	213
Canadian	8	4.9	155	95.1	163
Negro**	16	5.0	301	95.0	317

* Harris et al. (1963a) Ann. Hum. Genet. 26, 359. Majority of British samples were male. Tristan da Cunha islanders are an inbred population.

** Mostly male blood donors (see text).

TABLE 3

DISTRIBUTION OF C5 PHENOTYPES AMONG PHENOTYPES AT THE E_1 LOCUS FOR CHOLINESTERASE IN 2102 PARENTAL SERA.

E_1 Phenotype	C5+	C5-
Usual (U)	163	1877
Intermediate (I)	7	52
Atypical (A)	0	1
UF*	0	1
IF*	0	1

* Two phenotypes distinguished spectrophotometrically by unusual inhibition by sodium fluoride (see text).

TABLE 4

SECREGATION OF C5 PHENOTYPES.

Parental C5 phenotype ♂		♀	No. of families tested	Obs. or expd.	Progeny phenotype C5+ ♂	C5+ ♀	C5- ♂	C5- ♀
-	x	-	297	Obs.	1	1	571	559
				Expd.	0	0	575.2	556.8
+	x	-	85	Obs.	73	86	98	78
				Expd.(1)	87.3	83.7	83.7	80.3
				Expd.(2)	87.6	84.0	83.4	80.0
-	x	+	57	Obs.	49	46	66	51
				Expd.(1)	58.7	49.5	56.3	47.5
				Expd.(2)	58.5	49.4	56.5	47.6
+	x	+	12	Obs.	16	10	5	18
				Expd.(1)	16.0	21.3	5.0	6.7
				Expd.(2)	16.0	21.3	5.0	6.7

Expd.(1) Assumes frequency of E_2^+ is 0.0413 in both sexes from parental data

$\chi^2_6 = 33.7$, $P < 0.01$

Expd.(2) Assumes frequency of E_2^+ is 0.0477 from male parental data and 0.0349 from female parental data.

$\chi^2_6 = 33.7$, $P < 0.01$

TABLE 5

FERTILITY OF MATINGS BETWEEN DIFFERENT C5 PHENOTYPES.

Parental C5 phenotype ♂		♀	No. of families	Live children	Fetal deaths	Postnatal deaths
-	x	-	884	4763 (1)	831	1779
				5.39 (2)	0.94	2.01
+	x	-	85	455	93	166
				5.35	1.09	1.95
-	x	+	57	321	48	113
				5.63	0.84	1.98
+	x	+	12	77	19	30
				6.42	1.58	2.50

(1), (2) First row is total number, second row is number per family.

TABLE 6

AGE DISTRIBUTION OF PHENOTYPES FROM SEGREGATING MATINGS.

Parental C5 phenotype ♂		♀		Progeny phenotype C5+ ♀	C5+ ♂	C5- ♀	C5- ♂
+	x	-	Mean age, yrs.	9.05	8.09	9.66	10.11
			Total No. of Progeny	86	73	78	98
			No. 14 yrs. or older	22	10	20	28
			Propn. 14 yrs. or older	0.26	0.14	0.26	0.29
-	x	+	Mean age, yrs.	8.78	8.06	9.99	10.25
			Total No. of Progeny	46	49	51	66
			No. 14 yrs. or older	10	8	16	17
			Propn. 14 yrs. or older	0.22	0.16	0.31	0.26
+	x	+	Mean age, yrs.	8.93	9.75	11.79	13.80
			Total No. of progeny	10	16	18	5
			No. 14 yrs. or older	3	2	7	2
			Propn. 14 yrs. or older	0.30	0.13	0.39	0.40
Pooled			Mean age, yrs.	8.95	8.27	10.0	10.27
			Total No. of progeny	142	138	147	169
			No. 14 yrs. or older	35	20	43	47
			Propn. 14 yrs. or older	0.24	0.14	0.29	0.27

Fig. 1. Photograph of two gel slices each carrying three C5 phenotypes. Left gel (from left to right) C5-, C5+, C5-. Right gel (from left to right) C6+, C5-, C5-. Only the anodic side of the horizontal gel, run by the Honolulu technique, is shown. The point of sample insertion is indicated by horizontal arrows and the direction of migration towards the anode by a vertical arrow.

SECTION "D": COMPARATIVE CATTLE PHYSIOLOGY

Paper No.	Title	Describes: -
30	Comparative nitrogen digestibility in Brahman, Brahman x Shorthorn, Africander x Hereford, and Hereford steers. (1962)	-first published account of differences in efficiency of digestion between Bos taurus & Bos indicus cattle.
31	Weight gain and faecal nitrogen excretion in grazing British and zebu crossbred steers.	-relationships between weight gain and N digestion in grazing cattle.

J. Agric. Sci. (1962), 58, 333
Printed in Great Britain

Comparative nitrogen digestibility in Brahman, Brahman × Shorthorn, Africander × Hereford, and Hereford Steers

By G. C. ASHTON

Cattle Research Laboratory, C.S.I.R.O. Rockhampton, Queensland, Australia

(*Received* 30 *October* 1961)

Experimental work in the United States since 1930 has shown that calves with some Brahman blood grow faster to weaning, and that they grow faster as yearling and 2-year-old steers under summer grazing conditions, than do animals of British type (Warwick, 1958). Similar results have been obtained under Australian conditions. Thus at the National Cattle Breeding Station, Belmont, Rockhampton, Queensland, the following weights at 2½ years old are quoted as typical for animals under free-grazing conditions (Kennedy, 1961).

Shorthorn	802 lb.
Hereford	940 lb.
Africander × Shorthorn	1000 lb.
Africander × Hereford	1057 lb.
Brahman × Shorthorn	1104 lb.
Brahman × Hereford	1101 lb.

Birth weights for the Brahman crosses averaged about 90 lb., Herefords about 70 lb., and Africander crosses and Shorthorns about 64 lb.

A detailed description of the project has been published (Kennedy & Turner, 1959). It is aimed at the elucidation of factors causing superior growth rate in the cross-bred cattle, and subsequent investigation of such factors to assess their heredity. In general two complementary approaches are being made. First, all the known morphological and physiological differences between zebu and British breed cattle are being assessed for correlation with body-weight gain. Secondly, physiological factors known to influence growth rate are being investigated to determine whether the cross-breeds differ from the parental breeds.

In the second category gross nitrogen metabolism is being investigated. The gross nitrogen economy of a vertebrate can be considered to have three variable components, viz. nitrogen intake, efficiency of digestion and absorption of nitrogen, and efficiency of retention of absorbed nitrogen. Estimates of grazing intake are being made and will be reported later. Experiments are in progress to compare nitrogen retention efficiencies. A comparison of the efficiency of nitrogen digestion between breeds is reported in this paper.

MATERIALS AND METHODS

Animals

Steers from four breed groups were chosen as experimental animals. These were Grade Brahman (that is about seven-eights pure Brahman, graded up from Brahman males and Brahman × Shorthorn females), Brahman × Shorthorn first cross, Africander × Hereford first cross, and Hereford.

The distribution of the various steers used between experiments is shown in Table 1. The Brahman × Shorthorn, Africander × Hereford, and Hereford steers were all from the 1958 drop, i.e. were conceived in January or February 1958 and born at the end of the same year. The Grade Brahmans were, on average, about 3 months younger.

Diet

The diets presented to the steers were chosen to represent the type of feed and range of crude protein values available during the year to grazing animals on Belmont. The ration consisted of natural pasture hay, alone or in combination with lucerne hay. The components were chaffed individually and mixed in the appropriate proportions before feeding.

Either Bunch Spear grass (*Heteropogon contortus*) hay, crude-protein content between 4·6 and 5·3 %, or Queensland Blue grass (*Dicanthium sericeum*) hay, crude protein between 3·5 and 4·4 %, was used as representative of the natural pasture on Belmont. The lucerne hay used varied with batches between 15·5 and 17·4 % crude protein during the series of experiments. The composition of the ration in each experiment is shown in Table 1. Animals were fed according to metabolic body weight, that is body weight in kilograms to the power 0·73 (Blaxter, 1954) in runs 1 to 8. In runs 9 to 11, an equal amount of ration, irrespective of body weight, was fed.

Nitrogen balance

Because of the physical and psychological problems associated with beef cattle it is not possible to maintain steers in metabolism cages for both the equilibration and experimental periods. In the

Table 1. *Design of the nitrogen balance experiments*

Exp. no.	Date of Exp.	Ration: Composition	Ration: g. N/kg. B.W.$^{0.73}$	Ration: kg. D.M./kg. B.W.$^{0.73}$	Animals and breed groups	Average B.W.$^{0.73}$ kg.
1	Dec. 1959	One part lucerne hay; two parts spear grass	1·01	0·065	GB* 216, 218, 220, 223	44·7
2	Jan. 1960	One part lucerne hay; one part spear grass	1·69	0·073	BS 79, 103, 162, 183	56·0
3	Feb. 1960	Spear grass alone	0·55	0·065	AH 110, 116, 117, 358	50·5
					H 34, 88, 170, 207	45·7
4	May 1960	One part lucerne hay; two parts spear grass	1·16	0·072	GB 221, 228, 234, 236	60·9
5	June 1960	One part lucerne hay; one part spear grass	1·62	0·072	BS 146, 180, 200	7·35
					AH 125, 136, 197, 198, 214	68·7
					H 25, 41, 109, 157	58·4
6	Aug. 1960	One part lucerne hay; one part blue grass	1·29	0·072	GB 216, 218, 220, 223	52·8
7	Sept. 1960	One part lucerne hay; two parts blue grass	0·99	0·072	BS 79, 103, 162, 183	63·0
8	Sept. 1960	Blue grass alone	0·46	0·058	AH 110, 116, 117, 158	56·2
					H 34, 135, 155, 207	50·5
			g.N/day	kg. D.M./day		
9	June 1961	One part lucerne hay; one part blue grass	141·3	7·12	AH 110, 116, 117, 125	77·4
					H 25, 34, 109, 207	65·2
10	July 1961	One part lucerne hay; three parts blue grass	55·2	7·12	AH 110, 116, 117, 125, 197, 198,	78·3
					H 25, 34, 109, 207	67·0
11	Apr. 1961	Chaffed wheat straw	6·4	3·48	BS 162, 183	82·0
					AH 110, 158	69·7
					H 135, 207	66·7

* GB = grade Brahman; BS = Brahman × Shorthorn; AH = Africander × Hereford; H = Hereford; B.W. = body weight.

experiments reported here the steers were fed the appropriate ration for at least 14 days after the date when all the ration offered was consumed. The ration was fed in equal portions twice daily at 8 a.m. and 5 p.m. At the end of the equilibration period the animals were caged on Monday morning before the morning feed and released at the same time on Friday morning. Longer periods of confinement were found to cause weariness, and sometimes discomfort.

Animals were trained to the cages before the experiments began, and no difficulty with acclimatization was experienced, even with the quite temperamental zebu animals. The metabolism cages used were based on the design of Erwin, Dyer, Ensminger & Moore (1956). After initial positioning of the neck bail quantitative collection of faeces and urine was achieved readily.

Collection and storage of samples

The faeces and urine from each animal were removed and measured daily and a 10% aliquot of each taken and added to the previous day's aliquot. Analyses were carried out on the pooled aliquots.

The faeces were stored in heavy-gauge plastic bags kept in a refrigerator, and sealed to prevent moisture loss. It was found that these bags enabled very thorough mixing to be effected by pummelling and kneading the sealed bag. A quantity of pooled sample, usually between 500 and 600 g., was canned as described by Jacobsen, Kane & Flatt (1959). This was found to be an efficient method for preserving faeces for nitrogen and other analyses.

Urine was collected into 25 ml. of 9% $CuSO_4$. $5H_2O$ in 4 N–H_2SO_4. A daily 10% aliquot was stored in a sealed plastic bottle in a refrigerator. The pooled sample was deep frozen until analysed.

Methods of analysis

Fresh faeces were analysed for total nitrogen using a macroKjeldahl technique with mercuric oxide catalyst. Faecal nitrogen was fractionated in the following manner: 30 g. of faeces was weighed into a beaker, mixed well with 70 ml. of water and filtered through 'glass nylon' dress fabric. The excess liquid was squeezed out of the nylon filter, the residue transferred to the beaker, 50 ml. of water added and the process repeated. A final extraction with a further 50 ml. of water was made. The residue on the nylon sieve was washed copiously with tap water until the washings were clear, and then with 500 ml. of distilled water. The washed residue was transferred to a Kjeldahl flask and the nitrogen content determined, giving the 'undigested residue faecal nitrogen'.

The washings were transferred to a dialysis sac made of 1 in. diameter dialysis tubing, and the contents dialysed against running water for a minimum of 18 hr. After dialysis the contents of the sac were centrifuged at 2500 r.p.m. for 30 min. The supernatant was poured off into a Kjeldahl flask and the nitrogen content determined, giving the 'non-dialysable supernatant faecal nitrogen'. The sediment was similarly treated giving the 'non-dialysable sediment faecal nitrogen'. The difference between total faecal nitrogen', and undigested residue plus non-dialysable sediment and supernatant faecal nitrogen was considered to be the 'dialysable faecal nitrogen'.

Duplicate determinations of faecal nitrogen and faecal nitrogen fractions were made, and the duplicates were required to agree within ±1% of the mean value. Failing this a third determination was made with required tolerance of ±1% of two out of the three results, all three results being averaged. No difficulty was experienced in obtaining such precision, except with undigested residue nitrogen. Sometimes the mean of four determinations was used in deriving a value for this fraction.

Faecal dry matter was determined by oven drying at 110° C. overnight in triplicate.

RESULTS

Two sets of experiments were performed. In the first series (Exps. 1–8) the animals were fed according to body weight. In the second series (Exps. 9–11) the animals were fed equal amounts of ration regardless of body weight.

First series of experiments

In Exps. 1–3, four animals of each of the four breed groups, grade Brahman, Brahman × Shorthorn, Africander × Hereford and Hereford were used. In Exps. 4 and 5, different animals were used, four grade Brahman, three Brahman × Shorthorn, five Africander × Hereford, and four Hereford. In Exps. 6–8 the same animals were used as in Exps. 1–3, with the exception that two of the Herefords were replaced with two others (Table 1).

In the first series of experiments animals were fed on the basis of equal dry-matter intake per kg. body weight to the power 0·73. The composition, and hence nitrogen content, of the ration was varied between experiments (Table 1).

Table 2 shows average values for the animals in each breed group in each experiment for feed and total faecal nitrogen. The undigested residue faecal nitrogen values are shown for Exps. 4–8 only, as this analysis was not made on faecal samples from Exps. 1–3.

Table 3 shows various coefficients of digestibility derived from the data in Table 2.

Table 2. *Average values for animals in each breed group in Exps.* 1–8 *of feed, total faecal, and faecal undigested residue nitrogen*

	Nitrogen (g./day)											
	Grade Brahmans			Brahman × Shorthorn			Africander × Hereford			Hereford		
Exp. no.	Feed	Total faecal	Faecal undigested residue	Feed	Total faecal	Faecal undigested residue	Feed	Total faecal	Faecal undigested residue	Feed	Total faecal	Faecal undigested residue
1	44·8	20·2	—	56·8	26·0	—	50·2	23·3	—	47·2	22·2	—
2	74·6	22·1	—	91·4	29·8	—	83·6	25·8	—	75·5	25·5	—
3	24·9	18·7	—	32·1	23·7	—	28·6	21·6	—	26·0	19·9	—
4	69·5	27·2	9·2	84·6	32·0	10·8	79·7	29·7	9·5	67·5	29·7	8·8
5	97·0	29·8	10·7	118·2	32·5	12·1	111·3	33·4	12·0	94·2	30·9	10·6
6	65·3	24·0	6·4	79·6	30·4	9·1	70·5	25·6	7·6	64·0	26·1	6·5
7	50·0	22·4	5·9	60·9	28·4	8·2	53·9	26·7	7·3	49·0	23·4	6·2
8	22·3	16·7	4·8	27·1	20·7	7·0	24·3	18·6	5·8	21·8	17·2	4·9

Table 3. *Digestibility coefficients derived from data in Table* 2

	Grade Brahman				Brahman × Shorthorn				Africander × Hereford				Hereford			
Exp. no.	D.M.* dig. (%)	N† dig. (%)	Corrected N dig. (%)	N.R.N.‡ (g./100 g. D.M.)	D.M. dig. (%)	N dig. (%)	Corrected N dig. (%)	N.R.N. (g./100 g. D.M.)	D.M. dig. (%)	N dig. (%)	Corrected N dig. (%)	N.R.N. (g./100 g. D.M.)	D.M. dig. (%)	N dig. (%)	Corrected N dig. (%)	N.R.N. (g./100 g. D.M.)
1	58·8	54·9	—	—	57·7	54·2	—	—	58·3	53·6	—	—	56·1	53·0	—	—
2	61·0	70·4	—	—	57·4	67·4	—	—	58·1	69·1	—	—	57·6	66·2	—	—
3	55·1	24·9	—	—	52·1	26·2	—	—	53·6	24·5	—	—	52·5	23·5	—	—
Mean	58·3	50·1	—	—	55·7	49·3	—	—	57·7	49·1	—	—	55·4	47·6	—	—
4	58·1	60·9	86·8	0·410	56·4	62·2	87·2	0·401	58·5	62·7	88·1	0·408	53·7	56·0	87·0	0·497
5	56·8	69·3	89·0	0·435	59·8	72·5	89·8	0·385	58·0	70·0	89·2	0·433	58·5	67·2	88·7	0·483
Mean	57·5	65·1	87·9	0·423	58·1	67·4	88·5	0·393	58·3	66·4	88·7	0·421	54·8	61·6	87·9	0·490
6	55·6	63·2	90·2	0·463	51·6	61·8	88·6	0·468	54·1	63·7	89·3	0·446	53·0	59·2	89·8	0·538
7	54·6	55·2	88·1	0·444	51·3	53·4	86·4	0·439	53·6	50·5	86·6	0·456	54·1	52·2	87·5	0·473
8	48·0	25·1	78·3	0·427	45·7	23·6	74·2	0·388	46·0	23·5	76·2	0·401	4·33	21·1	77·6	0·428
Mean	52·7	47·8	85·5	0·445	49·5	46·3	83·0	0·432	51·2	45·9	84·0	0·434	50·1	44·2	85·0	0·480

* Dry-matter digestibility. † Apparent nitrogen digestibility. ‡ Non-residue faecal nitrogen.

Dry-matter digestibility

Dry-matter digestibility is the conventional relationship feed dry matter minus faecal dry matter divided by feed dry matter, expressed as a percentage. In each Exp. except 6 and 7 the Herefords exhibit the lowest dry-matter digestibility.

An analysis of variance of the data from Exps. 1–8 (Table 4) shows that the breed averages differed significantly ($P < 0.01$) in dry-matter digestibility.

The standard error of the mean dry-matter digestibility estimate for each breed group was calculated from the relationship

$$\sqrt{\left(\frac{2(\text{mean square for runs} \times \text{breeds})}{\text{No. of experiments}}\right)}.$$

The standard error works out at 0·63 dry-matter digestibility units. Multiplying by the appropriate value of t gives the value by which any two breed means must differ for the difference to be significant for a given probability. Table 5 shows the mean breed values for Exps. 1–8, and the differences between breed means for the six possible breed-group comparisons. From Table 5 it is seen that the mean dry-matter digestibility shown by the Hereford steers is very significantly lower ($P < 0.001$) than that for Grade Brahman and significantly lower than that for Africander × Hereford ($P < 0.05$). In addition the Brahman × Shorthorn steers were significantly poorer ($P < 0.01$) than the Grade Brahman steers.

Apparent nitrogen digestibility

Apparent nitrogen digestibility is the relationship feed nitrogen minus total faecal nitrogen divided by feed nitrogen, expressed as a percentage. This ratio takes no account of the heterogeneous origin of faecal nitrogen, but is commonly employed as a digestibility coefficient.

Table 3 shows that in each experiment except no. 7 the lowest mean apparent nitrogen digestibility is evidenced by the Hereford steers. Analysis of variance (Table 4) shows that the breed means differ significantly ($P < 0.01$). By the same statistical procedure used for interpreting the dry-matter

Table 4. *Analysis of variance of data in Table* 3

Variable	Source of variation	D.F.	Mean square	*F* value	S.E. of difference between breeds
Dry-matter digestibility (%)	Runs	7	69·1		
	Breeds	3	11·4	7·28**	0·63
	Runs × Breeds	21	1·57		
Apparent nitrogen digestibility (%)	Runs	7	1324·9		
	Breeds	3	16·6	8·50**	0·70
	Runs × Breeds	21	1·95		
Corrected nitrogen digestibility (%)	Runs	4	113·9		
	Breeds	3	1·3	0·19N.S.	—
	Runs × Breeds	12	6·8		
Non-residue faecal nitrogen (g. N per 100 g.) dry-matter intake	Runs	4	0·002662		
	Breeds	3	0·004371	11·15**	0·0125
	Runs × Breeds	12	0·000392		

N.S. Not significant. ** Significant at 1 % level.

Table 5. *Mean breed values for digestibility coefficients shown in Table* 3 *and significance of difference between breed means*

	Mean breed values, Exps. 1–8				Comparison of difference between breed means			
Variable	Grade Brahman	Brahman × Shorthorn	Africander × Hereford	Hereford		Brahman × Shorthorn	Africander × Hereford	Hereford
Dry-matter digestibility (%)	56·0	54·0	55·0	53·3	Grade Brahman	2·0**	1·0	2·7***
					Brahman × Shorthorn	—	1·0	0·7
					Africander × Hereford	—	—	1·7*
Apparent nitrogen digestibility (%)	52·99	52·66	52·20	49·80	Grade Brahman	0·33	0·79	3·19***
					Brahman × Shorthorn	—	0·46	2·86***
					Africander × Hereford	—	—	2·40**
Non-residue faecal nitrogen (g. N/100 g.) dry-matter intake	0·436	0·416	0·429	0·484	Grade Brahman	0·020	0·007	0·048**
					Brahman × Shorthorn	—	0·013	0·068***
					Africander × Hereford	—	—	0·055***

* Significant at 5 % level. ** Significant at 1 % level. *** Significant at 0·1 % level.

digestibilities it was demonstrated (Table 5) that the Hereford steers are very significantly inferior to the other three breed groups with regard to apparent nitrogen digestibility.

Corrected nitrogen digestibility

The nitrogen of the faeces in ruminants is derived from several sources. Faecal nitrogen includes the nitrogen of undigested feed residue, the nitrogen from digestive enzymes secreted into the alimentary tract, debris nitrogen from abraded epithelial or glandular cells, nitrogen from undigested rumen bacteria, digested nitrogen which has escaped absorption, and nitrogen trapped by bacteria proliferating in the lower gut. A true measure of digestion would involve distinction of the various faecal nitrogen components, but no separation scheme capable of unambiguous interpretation has yet been devised. The faecal nitrogen present when dietary nitrogen is absent is commonly termed metabolic faecal nitrogen. This is usually determined on nitrogen-free diets, but with ruminants such an approach is subject to criticism. Not only is it very difficult to persuade cattle to eat 'nitrogen-free' diets in sufficient quantity to meet their basal energy requirements, it is equally difficult to interpret the results because of the continual excretion during life of nitrogen-rich rumenal bacteria into the faeces.

In these experiments an attempt was made to distinguish the undigested feed residue nitrogen in the faeces from the remaining faecal nitrogen. This was done by sieving off the undigested feed residues. A similar procedure has been described by Mukherjee & Kehar (1949) who corrected their estimate of metabolic faecal nitrogen on low nitrogen feeds by allowing for the feed residues in the faeces. Using this procedure a 'corrected' nitrogen digestibility was determined in Exps. 4–8, using the relationship nitrogen in feed minus nitrogen in undigested faecal feed residues divided by nitrogen in feed, expressed as a percentage.

The mean values for each breed group in each experiment are shown in Table 3. There is no evidence of a consistent breed difference as with the dry matter or apparent nitrogen digestibilities, and an analysis of variance (Table 4) showed that the breed group means do not differ significantly.

It is not suggested that this sievable fraction represents the whole of the undigested hay in the faeces nor that the material sieved-off is free from bacterial or other occluded nitrogen. It is considered to represent a readily identifiable and reproducible fraction which is not concerned in the difference in nitrogen digestibility between breeds. In the second series of experiments described below further faecal fractionation techniques were applied in an attempt to identify a fraction responsible for the breed digestibility difference.

Remaining faecal nitrogen

The faecal nitrogen remaining after removal of the undigested residue nitrogen contains the fraction varying between breeds. Expressed in terms of dry-matter intake the Herefords excreted significantly more than the grade Brahmans or zebu cross-bred animals (Table 5). Because dry-mater intake was fixed according to metabolic body weight it is also true that the significant difference could be attributed to faecal nitrogen excreted per unit of metabolic body weight.

Second series of experiments

In Exps. 9 and 10 six Africander × Hereford and four Hereford steers were employed (Table 1). In these experiments the animals were fed the same amount of ration irrespective of body weight. In Exp. 11, three animals each of the breed groups grade Brahman, Brahman × Shorthorn, Africander Hereford, and Hereford were fed on the same quantity of chaffed wheat straw with a crude protein content of just under 1·0 %. Feed rejection disturbed the design of the experiment and only six animals (Table 1) completed the metabolism trial.

Table 6 shows the breed averages for each experiment for total nitrogen, undigested residue nitrogen, non-dialysable supernatant and sediment nitrogen, and dialysable nitrogen. In each experiment

Table 6. *Faecal nitrogen fractionation. Exps.* 9–11

Exp. no.	Breed group	Total	Nitrogenous faecal components (g. N/day)				Nitrogen in feed (g. N/day)
			Undigested residue	Non-dialysable supernatant	Non-dialysable sediment	Dialysable	
9	AH	51·22	6·38	19·25	14·23	11·36	141·26
	H	52·32	5·53	19·18	14·17	13·45	141·26
10	AH	39·16	4·73	16·23	10·12	8·08	55·18
	H	41·80	4·63	16·93	10·62	9·63	55·18
11	BS	14·97	1·98	8·74		4·25	6·40
	AH	15·01	2·00	8·74		4·27	6·40
	H	16·07	1·84	8·60		5·63	6·40

AH = Africander × Hereford; H = Hereford; BS = Brahman × Shorthorn.

the Africander x Hereford, and in Exp. 11 the Brahman x Shorthorn steers also, excreted less total faecal nitrogen than the Herefords. This nearly reaches significance at the 5% level. There was no significant difference between the breed groups in the amount of undigested residue nitrogen excreted, nor in the amounts of supernatant or sediment non-dialysable nitrogen. The amount of dialysable faecal nitrogen excreted by the Herefords was consistently greater than that by the Africander x Hereford, a highly significant difference ($P < 0.01$).

In Exp. 11 there was no difference between the Brahman x Shorthorn and Africander x Hereford steers in the amounts of the various faecal components excreted.

Dialysable faecal N and body weight

Table 7 shows the individual values for dialysable faecal nitrogen in Exps. 9–11 and the body weight of the steers in lb.

There was a significant negative correlation between body weight and dialysable faecal nitrogen in Exp. 9 ($r = -0.694$, $P < 0.05$) and in Exp. 10 ($r = -0.737$, $P < 0.05$). For the combined results of these two experiments $r = -0.807$ ($P < 0.001$). A similar but non-significant correlation was obtained in Exp. 11 ($r = -0.620$), but only six animals provided results in this experiment, the remainder having rejected the chaffed wheat straw offered.

The slope of the regression equation for each correlation was similar in each experiment and was −0·656, −0·620, and −0·696 g. dialysable faecal nitrogen per 100 lb. of body weight in Exps. 9–11 respectively. There was no evidence of a difference in slope between the breed groups in any of the experiments and the regression line appeared continuous between breeds. However, a larger number of animals covering a wider range of body weights would be required to confirm the continuity of the regression line.

Dialysable faecal nitrogen and faecal dry matter

Table 7 also shows the percentage of dry matter in the faeces in Exps. 9–11.

Dialysable faecal nitrogen and faecal dry matter were significantly negatively correlated in Exp. 9 ($r = -0.787$, $P < 0.01$), but not in Exp. 10 ($r = -0.574$). In Exp. 11 the correlation coefficient was positive, but not significantly so ($r = 0.257$). However, in this experiment comparison of the two Herefords showed that Hereford 135 (Table 7) excreted less dialysable nitrogen but had a greater percentage of dry matter in the faeces than Hereford 207. Similarly Africander x Hereford 158 excreted less dialysable nitrogen and had drier faeces than Africander x Hereford 110. The pair of Brahman x Shorthorn steers did not comply.

DISCUSSION

Very few direct comparisons between cattle breeds with respect to their relative digestive powers have been made. Several workers some years ago (for example, Matson, 1928; Warth & Gossip, 1930; Sayer, 1934) stated that the Indian zebu in its native habitat had superior digestive capacity for dry matter compared with figures which had at that time been published for European cattle breeds. Their conclusions were not based on direct comparison of individuals from the different breeds at the same time. French (1940) carried out the first comparative experiments, between African zebu and grade Ayrshire cattle (3/4 or 7/8 Ayrshire, 1/4 or 1/8 zebu). Although in seven out of ten experiments the small number of animals tested

Table 7. *Body weight* (B.W.) *in lb., dialysable faecal nitrogen* (D.F.N.) *g/day, and percentage faecal dry matter* (% D.M.) *in Exps.* 9–11

Breed	Animal no.	Exp. 9			Exp. 10			Exp. 11		
		B.W.	D.F.N.	%D.M.	B.W.	D.F.N.	%D.M.	B.W.	D.F.N.	%D.M.
AH	110	850	12·30	19·96	837	9·06	18·13	772	4·37	18·54
	116	784	12·64	19·60	794	8·17	19·26	—	—	—
	117	676	10·65	19·41	698	9·77	19·45	—	—	—
	125	965	10·60	18·97	977	8·25	18·17	—	—	—
	158	—	—	—	—	—	—	704	4·19	18·73
	197	969	11·39	20·65	962	8·04	17·42	—	—	—
	198	910	10·58	20·67	932	5·17	20·70	—	—	—
H	25	714	12·71	18·52	746	8·92	16·45	—	—	—
	34	589	15·02	16·53	634	9·88	17·13	—	—	—
	109	660	12·82	18·39	683	10·32	17·55	—	—	—
	135	—	—	—	—	—	—	692	4·92	22·69
	207	732	12·15	18·35	731	9·39	19·05	696	6·34	19·58
BS	161	—	—	—	—	—	—	768	4·70	19·33
	183	—	—	—	—	—	—	960	3·80	18·48

AH = Africander x Hereford; H = Hereford; BS = Brahman x Shorthorn.

gave clear indications of the superiority of the zebus with regard to crude-protein digestibility, French stated that his experiments did not support the conclusion that the zebus had superior digestive powers compared with his grade Ayrshires. In two out of the three experiments in which the grade Ayrshire cattle differed little from or were superior to the zebu cattle the feed consisted of concentrate mixture or maize meal. In the seven experiments, in which the zebus showed superior digestive capacity for nitrogen the feed was largely fibrous in nature. Later experiments (Duckworth, 1946; French, 1956) showed differences favouring the zebu, but the differences found were not considered to be of practical significance.

No direct comparison of the digestibility of zebu or zebu cross-bred beef cattle with European beef cattle seems to have been made prior to this study.

The results obtained in these experiments show that the amount of faecal nitrogen excreted on a given ration was greater from Herefords per unit of dry-matter intake, than from grade Brahman, Brahman × Shorthorn, or Africander × Hereford steers. The difference resides in the dialysable component of the faecal nitrogen. Because of the heterogeneous origin of faecal nitrogen components it is not easy to interpret the results of fractionation procedures. The dialysable fraction is clearly not undegraded food residues, nor is it undigested digestive enzymes, undegraded bacteria, or other protein or polypeptide material. It could be of either metabolic or food origin, and could vary between breeds and individuals because of its differential secretion or because of differential absorption of some of the end-products of digestion.

It is difficult to accept that the lower excretion of dialysable faecal nitrogen by heavier animals is a consequence of their greater body weight. It is reasonable to assume that the lighter animals are lighter, i.e. have grown more slowly, because they excrete more dialysable faecal nitrogen than heavier animals on the same intake. The possibility exists that the dialysable nitrogen is valueless to the animal. This would imply a compensatory excretion of the valueless nitrogenous material in the urine of the heavier animals. However, there is no evidence for this from urine analysis. The available evidence suggests that the dialysable faecal nitrogen represents a loss of nitrogenous material potentially capable of being converted by the animal into body tissue.

As stated above the difference in dialysable faecal nitrogen between animals could be due to differences in the efficiency of removal of the end-products of digestion from the alimentary tract, or to differential production of material secreted into the gut and not readily resorbed. If larger animals are more efficient absorbers of digesta this would perhaps fit the observation that the dry-matter content of zebu cross-bred faeces is consistently higher than that of Herefords on a given diet (Ashton & Kennedy, 1962), and also the observations of Quarterman, Phillips & Lampkin (1957), on differences in osmotic pressure in the digestive tracts of zebu and European cattle. Further support comes from the significant negative correlation ($r = -0{\cdot}787$) between faecal dry matter and dialysable faecal nitrogen in Exp. 9 (Table 7). A similar effect is shown in Exp. 10, although it is not significant. On a very low nitrogen intake (Exp. 11) the between breed correlation is not significant and is positive. However, within breeds the Africander × Hereford and Herefords support the observations in Exps. 9 and 10 that animals with drier faeces excrete less dialysable nitrogen, while the Brahman × Shorthorn steers do not.

On the other hand there is some evidence that the difference in dialysable faecal nitrogen in the faeces is due to differential secretion of nitrogenous matter into the gut. In Exp. 11 (Table 6) the Herefords are excreting more dialysable nitrogen in their faeces (5·63 g.) than the amount of food nitrogen degraded in the tract, i.e. 6·40 g. feed nitrogen less 1·84 g. undigested residue nitrogen.

The nature of the mechanism causing the difference in dialysable nitrogen excretion bears on the question of whether the dialysable nitrogen is of feed or metabolic origin. The origin of this nitrogen is important in interpreting the value of its loss to the animal (see below). It is possible that the dialysable faecal nitrogen originates from ingested material directly, without entering the body and being subsequently secreted as metabolic nitrogen. The evidence for this is that dialysable faecal nitrogen varies with nitrogen intake (Exps. 9–11). However, this does not preclude the possibility that the secretion of digestive enzymes is proportional to the amount of food nitrogen in the tract. If these enzymes were not completely re-absorbed from the lower gut but appeared as dialysable nitrogen, then a correlation between dialysable nitrogen and feed nitrogen would be expected. The results from the Herefords in Exp. 11, considered above, lend support to the metabolic rather than direct food origin of the dialysable faecal nitrogen.

Significane of difference in dialysable faecal nitrogen excretion

In Exps. 9 and 10 the mean difference between the Africander × Hereford and Herefords in dialysable faecal nitrogen was $1{\cdot}81 \pm 0{\cdot}51$ g. per day. This difference is confirmed in the results of Exps. 1–8 in which the grade Brahman and Hereford steers, being of similar body weight, were fed similar rations. The difference between these breeds in total faecal nitrogen averaged 1·73 g.

The Africander × Hereford and Hereford steers in Exp. 9 and 10 at about 30 months of age had mean body weights of 863 and 686 lb., respectively. It can be calculated from data on the nitrogen content of live-weight gain at various stages of growth (Mitchell, 1929) that this difference in live-weight would be equivalent to about 2300 g. nitrogen. If the Africander × Hereford steers had saved 1·81 ± 0·51 g. nitrogen per day throughout their growth life of 900 days this would represent 1630 ± 460 g. nitrogen. If the dialysable faecal nitrogen was of metabolic origin it would almost certainly represent a loss of fully utilizable nitrogen, and would then account for about 70 ± 20 % of the difference in storage between the breeds. If the dialysable nitrogen originated directly from ingested pasture the value of the loss would depend on the nature of the nitrogen and hence its efficiency of utilization.

It appears, from Exp. 9 and 10, that the same kind of difference in digestive function may apply, to a lesser degree, between animals within breeds as well as between breeds. Within breeds the correlation between dialysable faecal nitrogen and body weight is −0·593 (7 D.F. $0·05 < P < 0·1$). The correlations between gain and dialysable faecal nitrogen when compared between and within breeds are sufficiently similar that the highly significant breed difference in body weight disappears when adjusted for covariation with dialysable faecal nitrogen.

Taking the mean results for all animals of both breed groups in Exps. 9 and 10, the correlation between dialysable faecal nitrogen and body weight is −0·807. This implies that $-0·807^2 = 65$ % of the variation in body weight is accounted for by variation in dialysable faecal nitrogen. This estimate is similar to that derived above by different means, for the proportion of the breed difference in body weight accounted for by dialysable faecal nitrogen.

SUMMARY

1. Eight nitrogen metabolism experiments, each with sixteen steers representing grade Brahman, Brahman × Shorthorn, Africander × Hereford and Herefords, were carried out. The steers were fed according to body weight to the power 0·73, on rations consisting of natural pasture hay alone or in combination with lucerne hay.

2. These experiments showed that the steers with zebu blood were significantly superior to the Herefords with regard to dry-matter digestibility and apparent nitrogen digestibility. The breed difference disappeared when nitrogen digestibility was calculated from the relationship feed nitrogen minus undigested faecal residue nitrogen, divided by feed nitrogen.

3. Two further nitrogen metabolism experiments with six Africander × Hereford and four Hereford steers fed natural pasture hay plus lucerne hay, and one experiment with two Brahman × Shorthorn, two Africander × Hereford and two Hereford steers fed chaffed wheat straw were carried out. The steers were fed an equal amount of ration, irrespective of body weight.

4. In these three experiments faecal nitrogen was fractionated into undigested residue nitrogen, non-dialysable centrifugable nitrogen, non-dialysable non-centrifugable nitrogen, and dialysable nitrogen. The breed groups differed significantly in the amount of dialysable faecal nitrogen excreted but not in any other component.

5. The amount of dialysable faecal nitrogen excreted on equal intake was very significantly negatively correlated with body weight ($r = -0·807$, $P < 0·01$).

6. It was found that in two experiments the mean difference between Africander × Herefords and Hereford steers, mean body weights 863 and 686 lb., respectively, was 1·81 ± 0·51 g. nitrogen per day. It was calculated that this would represent about 70 ± 20 % of the body-weight difference between the breeds if the lost nitrogen was fully utilizable. This agreed well with the estimate of 65 % derived from the square of the correlation coefficient.

I am grateful to Mr H. G. Turner, Officer-in-Charge, Cattle Research Laboratory, for helpful suggestions during the course of this work, to Mr J. F. Kennedy, Officer-in-Charge, National Cattle Breeding Station, 'Belmont', and his staff for day to day management of the metabolism experiments, and to Misses C. Cruden, G. Perkins, and C. Steer for technical assistance.

REFERENCES

ASHTON, G. C. & KENNEDY, J. F. (1962). *Aust. J. Agric. Res.* In preparation.

BLAXTER, K. L. (1954). In *Progress in the Physiology of Farm Animals*, ed. Hammond, J. **1**, 3. London: Butterworth.

DUCKWORTH, J. (1946). *Trop. Agriculture, Trin.* **23**, 4.

ERWIN, E. S., DYER, I. A., ENSMINGER, M. E. & MOORE, W. (1956). *J. Anim. Sci.* **15**, 435.

FRENCH, M. H. (1940). *J. Agric. Sci.* **30**, 503.

FRENCH, M. H. (1956). *Emp. J. Exp. Agric.* **24**, 235.

JACOBSEN, W. C., KANE, E. A. & FLATT, W. P. (1959). *J. Anim. Sci.* **18**, 650.

KENNEDY, J. F. (1961). Breed variation in rates of growth of cattle in the tropics. Communication to Section L of 35th Congress of A.N.Z.A.A.S., Brisbane.

KENNEDY, J. F. & TURNER, H. G. (1959). A project on genetics of adaptation in cattle. *C.S.I.R.O., Aust. Div. Anim. Hlth, Prod., Div. Rep.* no. 8 (Series SW–3).

MATSON, J. (1928). *J. Cent. Bur. Anim. Husb. India*, **2**, 83.

MITCHELL, H. H. (1929). *Bull. Nat. Res. Counc.* no. 67.

MUKHERJEE, R. & KEHAR, N. D. (1949). *Indian J. Vet. Sci.* **19**, 99.

QUARTERMAN, J. PHILLIPS, G. D. & LAMPKIN, G. H. (1957). *Nature, Lond.*, **180**, 552.

SAYER, W. (1934). *Agric. Live-stock India* **4**, 105.

WARTH, F. J. and GOSSIP, F. T. (1930). *Mem. Dep. Agric. India*, **10**, 1.

WARWICK, E. J. (1958). *J. Anim. Sci.* **17**, 922.

(31)

Reprinted from the
AUSTRALIAN JOURNAL OF AGRICULTURAL RESEARCH
VOLUME 14, NUMBER 6, PAGES 898–908, NOVEMBER 1963

WEIGHT GAIN AND FAECAL NITROGEN EXCRETION IN GRAZING BRITISH AND ZEBU CROSSBRED STEERS

By G. C. ASHTON

Reprinted for the
Commonwealth Scientific and Industrial Research Organization
Australia

WEIGHT GAIN AND FAECAL NITROGEN EXCRETION IN GRAZING BRITISH AND ZEBU CROSSBRED STEERS

By G. C. ASHTON*

[*Manuscript received January* 31, 1963]

Summary

The excretion of faecal nitrogen by 16 grazing steers, eight Hereford × Shorthorn and eight Zebu crossbreds, was determined at intervals over a period of 11 months. It was found that the non-dialysable faecal nitrogen fraction was the one most strongly correlated with gain.

Statistically this fraction accounted for 68% of the variation in average gain of the two breed groups between measurement intervals, and for 75% of the variation in weight gain between individual steers during the summer gain period.

The data gave no indication of inherent breed differences in intake, as judged by non-dialysable nitrogen excretion.

I. INTRODUCTION

It has been found at the National Cattle Breeding Station at "Belmont," that under free grazing on Queensland native pastures Zebu crossbred steers grow faster than Hereford or beef Shorthorn steers (Kennedy, unpublished data, 1961). An objective of the conjoint programme of this Station and the Cattle Research Laboratory is to identify the physiological factors responsible for the difference in weight gain, with a view to using such factors as selection criteria in a breeding programme (Kennedy and Turner 1959).

One approach is to investigate the factors known to control growth rate in mammals, and ascertain whether the breed groups differ with regard to response to these factors. It is also relevant to determine the importance of a given factor with regard to its contribution to differences in weight gain between individuals within a breed. In this way the various factors may be accorded proper emphasis in a selection programme.

Ruminants are known to differ in intake while grazing, and this is an important source of variation causing differences in growth rate between individuals. Unfortunately the problems involved in the unequivocal measurement of nitrogen intake in grazing animals have not been solved (Ivins 1959), and consequently the percentage of difference in growth rate due to differences in intake of nitrogen is not measurable directly. It is known that faecal nitrogen excretion and nitrogen intake are correlated. However, the diversity of relationships quoted in the literature is an indication of the lack of fundamental knowledge in this field (Forbes 1950; Lancaster 1954; Raymond *et al.* 1954; Milford 1957; Hutchinson 1958; Lambourne 1958).

* Division of Animal Genetics, C.S.I.R.O., Cattle Research Laboratory, Rockhampton, Qld.

The first objective of the experiments reported in this paper was to establish the relationship between faecal nitrogen excretion and weight gain in grazing steers on "Belmont", and to determine the extent to which differences in gain are associated with differences in faecal nitrogen excretion. The second objective was to compare the relative faecal nitrogen excretion of the two breed groups in different seasons.

To assist the interpretation of faecal nitrogen excretion, faeces were fractionated into three fractions, viz. non-dialysable nitrogen, dialysable nitrogen, and non-sievable residue nitrogen, as it has been shown previously that these fractions show different relationships with body weight and gain in stall-fed animals (Ashton 1962).

II. Methods

(*a*) *Animals*

A group of 16 steers was used in these experiments. All the steers were born in November or December 1959, at the National Cattle Breeding Station, "Belmont", near Rockhampton, Qld. Details of the management, environment, and composition of the herd have been presented by Kennedy and Turner (1959).

Table 1

DATES OF FAECAL COLLECTIONS, CORRESPONDING AVERAGE BODY WEIGHTS FOR EACH BREED GROUP, AND MEAN DAILY GAIN BETWEEN EXPERIMENTS

Date of Weighing and Start of Faecal Collection	Mean Body Weight (lb)			Days between Expts.	Gain between Expts. (lb/day)		
	British Breed	Brahman × Hereford	Africander × Hereford		British Breed	Brahman × Hereford	Africander × Hereford
Apr. 22, 1961	512	698	584	25			
May 17, 1961	546	739	623	45	1·36	1·63	1·56
July 1, 1961	543	746	635	52	−0·07	0·16	0·27
Aug. 22, 1961	502	720	599	15	−0·79	−0·50	−0·64
Sept. 6, 1961	510	713	595	28	0·53	−0·47	−0·27
Oct. 4, 1961	525	708	615	49	0·54	−0·18	0·36
Nov. 22, 1961	508	680	587		−0·35	−0·57	−0·57
Dec. 12, 1961	575	754	659	20	3·35	3·70	3·60
Jan. 19, 1962	614	812	718	38	1·03	1·53	1·55
Feb. 19, 1962	696	916	794	31	2·65	3·35	2·45
Mar. 16, 1962	742	967	841	25	1·84	2·04	1·88

Eight of the steers were the progeny of Hereford × Shorthorn cows and Hereford × Shorthorn bulls, i.e. representatives of an F_2 generation from original Hereford × Shorthorn matings. These animals are referred to as "British" steers.

Of the remaining eight animals, four were the progeny of Hereford cows and Brahman bulls and are referred to as "Brahman × Hereford" steers, and four were the progeny of Hereford cows and Africander bulls and are referred to as "Africander

× Hereford" steers. The Brahman × Hereford and Africander × Hereford steers collectively are referred to as "Zebu crossbred" steers.

(*b*) *Experimental Treatment*

The steers were weaned in August 1960 and were then run as one group, being subjected to management manipulations concurrently. At intervals (see Table 1) commencing in April 1961 the steers were mustered, weighed, and transferred to individual concrete-floored pens. The steers remained in these pens for 48 hr, during which period no food was offered but water was available.

During the 2 days faecal samples were collected from the droppings. As far as possible a sample weighing about 500 g was taken from each freshly voided dung pat; urine contamination was avoided. The dung pat samples were stored in plastic bags, one for each steer, and held in a refrigerator until analysed.

During each day, at intervals, the remaining faeces were removed from each stall, placed in a receptacle, and subsequently weighed. A sample was taken for organic matter determination ("floor sample").

(*c*) *Analysis of Faecal Samples*

The well-mixed dung pat samples were analysed in triplicate by the following procedures. A macro-Kjeldahl technique employing mercuric oxide catalyst was used for determining nitrogen.

Total Nitrogen.—A 10 g sample was digested directly.

Non-Sievable Residue Nitrogen.—A 30 g sample was transferred to a sieve of "glass Nylon" dress fabric, 112 × 112 meshes per inch, of pore size 0·185 mm square, and washed with a tap-water spray for 30 min. The nitrogen content of the residue was determined.

Non-Dialysable Nitrogen.—A 20 g sample was transferred with 100 ml of water to a 1 in. diameter dialysis tube, sealed at both ends, and placed overnight in running tap-water. The nitrogen content of the tube was determined. This value minus the value for residue nitrogen gave non-dialysable nitrogen.

Dialysable Nitrogen.—This was obtained by subtracting the value for dialysis tube contents from that for total nitrogen.

Organic Matter.—Organic matter was determined in triplicate on 15 g samples by drying overnight at 105°C, cooling, weighing, and then correcting the dry weight for the weight of ash produced after heating in a muffle furnace at 800°C for 4 hr. The dry weight of non-sievable residue was determined in triplicate on 30 g samples of faeces, after sieving through "glass Nylon" dress fabric and drying the well-washed residue at 105°C.

Computation of Daily Outputs.—The total weight of organic matter was obtained from the organic matter of the dung pat samples plus the organic matter of the floor samples. The daily output of nitrogen fractions was derived from the relationship:

$$\frac{24}{48} \times \frac{\text{Total weight of organic matter}}{\text{Organic matter in dung pat samples}} \times \left(\frac{\text{Weight of N fraction}}{\text{in dung pat sample}}\right).$$

(*d*) *Verification of Technique*

The relationship between the daily output of faecal components estimated from dung pat samples, and the true daily output estimated from complete collection, was assessed by experiments with animals in metabolism cages. The average excretion of total faecal nitrogen from 11 such animals over 4 days by the complete collection technique was 28·82 g/day. The corresponding figure for dung pat samples was 28·34 g/day. The standard error of the difference was $\pm$ 0·55 g.

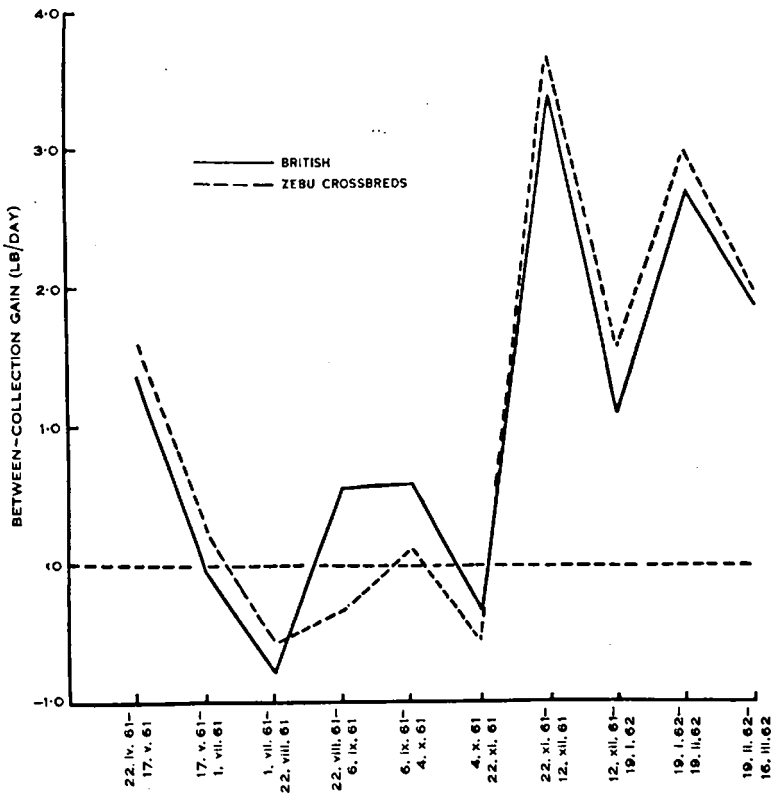


Fig. 1.—Mean weight gains between collection periods for British (solid line) and Zebu crossbred (broken line) steers. Horizontal axis, between-collection period; vertical axis, weight gain (positive) or weight loss (negative).

III. Results

The chief interest in the results was to determine the relationship between the excretion of faecal nitrogen components and weight gain, both between animals within breeds, and between breeds. The data have been examined in two ways. Firstly, for the steers in each breed group, the correlation between the average excretion of faecal component during a collection period, and the average weight gain in the succeeding between-collection interval, has been calculated. Secondly, the

correlations between overall gain for individual steers, and the total excretion of faecal component by each steer during two seasons, have been calculated.

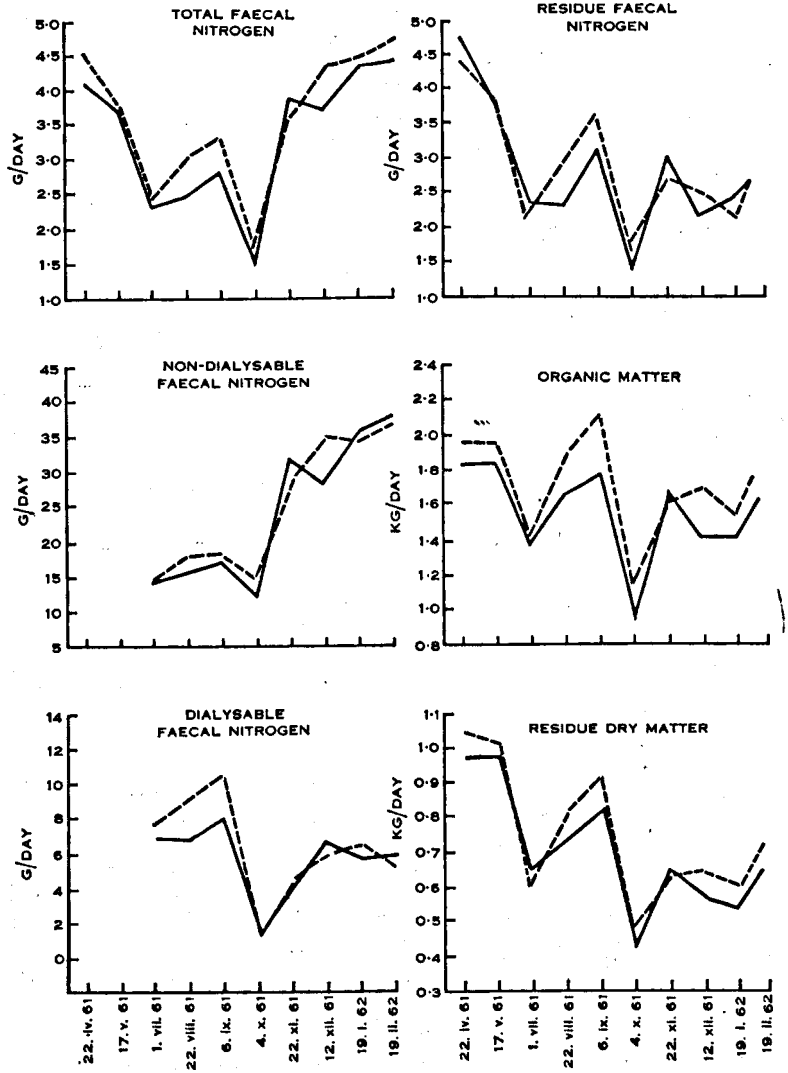


Fig. 2.—Excretion of faecal components during collection period by British (solid line) and Zebu crossbred (broken line) steers. Horizontal axis in each case, date of measurement; vertical axis, average daily excretion of faecal component during collection period.

(*a*) *Correlations between Faecal Component Excretion in Individual Collection Periods and Gain between Succeeding Collections*

(i) *Change in Body Weight of Steers*

The mean body weights for the British, Africander × Hereford, and Brahman × Hereford steers throughout the experiment are shown in Table 1. More informa-

tive is the change in body weight between collection periods expressed as gain in pounds per day (Table 1 and Fig. 1). This shows the typical pattern observed under Queensland native pasture grazing conditions, that is, weight gain in the summer months and no gain, or even weight loss, during winter. During the 11 months of the experiment the Brahman × Hereford steers gained on an average 269 lb, the Africander × Hereford 257 lb, and the British breed steers 230 lb. These differences in weight gain were not significant, although the order is similar to that previously observed on "Belmont". There was a tendency, just significant, for Zebu crossbreds to gain more than British breed steers during summer, and less during winter. The Zebu crossbreds gained significantly better ($P < 0{\cdot}01$) from weaning in August 1960 to March 1962 than did the British steers, but the difference in gain between the Africander × Hereford and Brahman × Hereford steers in this period was not significant. This is typical of previous observations comparing the growth rate of Zebu crossbred steers with those of Shorthorn, Hereford, and Shorthorn × Hereford steers on "Belmont" from weaning to slaughter (Kennedy 1961).

TABLE 2

CORRELATIONS BETWEEN AVERAGE BREED VALUES FOR FAECAL NITROGEN COMPONENTS FOR EACH COLLECTION, AND CORRESPONDING GAINS (LB/DAY) DURING THE FOLLOWING BETWEEN-COLLECTION PERIOD

Faecal Nitrogen Component	Breed Group Correlation			Pooled Correlation Coefficient
	British Breed	Brahman × Hereford	Africander × Hereford	
Total	0·736*	0·685*	0·651*	0·689**
Non-dialysable	0·877**	0·833**	0·774*	0·826**
Dialysable	−0·091	−0·176	−0·247	−0·179
Residue	0·197	0·042	0·136	0·120

*$P < 0{\cdot}05$. **$P < 0{\cdot}01$.

(ii) *Excretion of Total and Non-Dialysable Faecal Nitrogen*

The average daily outputs of total faecal nitrogen and non-dialysable faecal nitrogen for the British and Zebu crossbred breed groups are shown in Figure 2. The correlations between average daily gain (lb/day) and total faecal nitrogen for the three breed groups are shown in Table 2. In each case the correlation coefficient was significant ($P < 0{\cdot}05$). There were no significant differences between breeds in the value of the regression coefficients. After correcting for between-breed variation, a pooled coefficient of 0·689 ($P < 0{\cdot}01$) was obtained for correlation between daily total faecal nitrogen excretion and daily weight gain in the next between-collection period.

No values for non-dialysable faecal nitrogen were obtained in the first two collection periods. The simple correlation coefficients derived from the relationships between non-dialysable nitrogen excretion and daily weight gain in the next between-

collection period for each breed group are shown in Table 2. The values of the individual correlation coefficients were significant, there were no significant differences between breed groups in the value of the regression coefficients, and the pooled correlation coefficient, corrected for between-breed variation, was 0·826 ($P < 0·01$).

TABLE 3

AVERAGE DAILY EXCRETION OF NON-DIALYSABLE NITROGEN (NDN), RESIDUE NITROGEN (RN), AND DIALYSABLE NITROGEN (DFN) FOR TWO PERIODS FOR EACH STEER, AND CORRESPONDING MEAN BODY WEIGHT AND AVERAGE DAILY GAIN

Steer No.	July to October					November to February				
	Excretion (g/day)			Mean Body Wt. (lb)	Av. Daily Gain (lb/day)	Excretion (g/day)			Mean Body Wt. (lb)	Av. Daily Gain (lb/day)
	NDN	RN	DFN			NDN	RN	DFN		
Hereford × Shorthorn										
1	15·2	2·1	4·9	532	−0·24	33·4	2·6	6·0	649	2·05
2	16·5	2·5	6·4	510	−0·22	36·2	2·7	6·0	627	2·06
3	14·1	2·2	5·1	497	−0·16	28·9	2·3	2·9	623	2·06
4	15·6	2·4	5·8	583	−0·35	34·1	2·7	5·9	706	2·19
5	13·9	2·3	5·2	509	−0·18	35·0	2·6	4·2	625	1·88
6	15·5	2·3	5·8	543	−0·16	36·9	2·9	5·5	691	2·48
7	14·5	2·1	5·5	480	−0·28	33·3	2·6	7·0	589	2·04
8	12·5	2·0	5·8	443	−0·35	22·8	2·0	4·5	515	1·64
Brahman × Hereford										
9	15·4	2·4	8·1	668	−0·54	33·8	2·4	7·3	765	2·37
10	17·6	2·6	7·7	688	−0·22	41·8	2·8	6·1	822	2·25
11	19·2	3·0	8·2	791	−0·61	54·8	3·5	7·7	924	3·12
12	15·7	2·6	6·8	708	−0·47	40·9	2·6	5·0	797	2·34
Africander × Hereford										
13	14·0	2·3	5·1	560	−0·22	29·4	2·0	3·8	664	2·11
14	17·0	2·8	6·6	674	−0·34	39·7	2·8	4·1	784	2·25
15	14·9	2·2	6·2	575	−0·37	40·3	2·6	5·6	721	2·30
16	15·7	2·4	7·7	604	−0·40	39·6	2·7	5·1	725	2·26

(iii) *Excretion of Residue Faecal Nitrogen, Faecal Organic Matter, and Residue Faecal Dry Matter*

The shape of the plot of the excretion of residue faecal nitrogen against time (Fig. 2) differs from that of gain *v.* time (Fig. 1). Whereas between-collection gain was greatest in the November–December period, the excretion of residue faecal nitrogen was relatively low at this time. The pooled correlation between daily gain and daily excretion of residue faecal nitrogen, corrected for between-breed variation, was 0·120, a non-significant value (Table 2).

The excretion of residue dry matter was highly correlated with the excretion of residue faecal nitrogen (Fig. 2), and not significantly correlated with between-

collection gain. The excretion of organic matter similarly was not significantly correlated with between-collection gain.

(iv) *Excretion of Dialysable Faecal Nitrogen*

The daily excretion of dialysable faecal nitrogen for the British and Zebu cross-bred steers during each collection period is also shown in Figure 2. The excretion of this component is clearly not associated with subsequent gain, the excretion in the winter months of July and August exceeding that in the summer months of December, January, and February. This is shown formally by the non-significant, negative values of the simple correlation coefficients (Table 2).

TABLE 4

SIMPLE CORRELATIONS BETWEEN METABOLIC BODY WEIGHT ($W^{0.73}$), AVERAGE DAILY GAIN, AND DAILY EXCRETION OF FAECAL NITROGEN COMPONENTS, IN TWO PERIODS CALCULATED OVER ALL ANIMALS

Variables Correlated	Period	Gain and Weight Correlations	Correlations with Nitrogen Components		
			Non-Dialysable	Residue	Dialysable
Gain and weight	July to October	−0·681**			
	November to February	−0·868**			
Excretion and weight	July to October	—	0·816**	0·869**	0·908**
	November to February	—	0·876**	0·698**	0·121
Excretion and gain	July to October	—	−0·418	−0·508*	−0·722**
	November to February	—	0·867**	0·769**	0·544*

*$P < 0·05$. **$P < 0·01$.

(*b*) *Correlations between the Faecal Nitrogen Excretion of Individual Steers and their Weight Gain in the Winter and Summer Periods*

To permit valid comparisons, analysis has been confined to the results for the last eight collections, for which data on all fractions are available. The first four of these collections were made during a time of slight loss in body weight, averaging − 0·32 lb/day, the last four during a time of steady growth, averaging 2·21 lb/day. These two nutritionally distinct seasons, July–October and November–February, corresponding approximately to winter and summer respectively, have been treated separately. Table 3 shows the average daily faecal nitrogen excretion of each steer during the two periods, and the corresponding average body weight and daily weight gain.

Simple correlation coefficients calculated over all animals, showing the relationships in both periods between faecal nitrogen components and gain, between gain and metabolic body weight (i.e. $BW^{0·73}$), and between excretion and body weight, are shown in Table 4.

The excretion of non-dialysable nitrogen is significantly correlated with gain in November–February but not in July–October. Residue nitrogen is significantly correlated with gain in November–February and negatively correlated with gain in July–October. Dialysable nitrogen is less strongly correlated with November–February gain, but strongly negatively correlated with July–October gain.

Body weight and the excretion of non-dialysable and residue nitrogen were strongly correlated in both periods, while dialysable nitrogen was correlated with body weight in July–October only.

Gain and body weight were significantly correlated, positively in November–February, negatively in July–October.

IV. Discussion

Evidence is accumulating that the three components of faecal nitrogen studied here, that is, dialysable nitrogen, non-dialysable nitrogen, and residue nitrogen, probably represent different, but not necessarily discrete, physiological entities. Thus dialysable faecal nitrogen is differentially excreted by British and Zebu cross-bred beef steers (Ashton 1962), and also differs from non-dialysable and residue faecal nitrogen in its relationships with faecal water (unpublished observations). The results in Figure 2 and Table 2 further emphasize the relative independence of these three fractions, the pattern of excretion of the three components during the period of the experiment being dissimilar.

(*a*) *Relationship between Non-dialysable Nitrogen and Gain*

Of the three faecal nitrogen fractions studied, non-dialysable nitrogen is clearly the best indicator of succeeding weight gain in grazing animals. Within a group of grazing animals at a given time, residue nitrogen is also a reasonably efficient indicator, but its dependence on season, presumably as a result of variation in pasture quality, reduces its value. Dialysable nitrogen is not a reliable indicator of gain in growing, grazing animals.

In interpreting the extent to which variation in non-dialysable nitrogen excretion is related to gain, it is important to distinguish between the extent to which the variation statistically accounts for gain and the extent to which it is responsible for differences in gain.

From Table 2 it is evident that the correlations between non-dialysable nitrogen excretion and gain for the three breed groups are similar, and are not significantly different from the pooled correlation coefficient of 0·826. Accordingly, on a statistical basis, 68% of the variation in average weight gain of the breeds between collections is accounted for by variation in the excretion of non-dialysable nitrogen. Similarly, from Table 4 the correlation between non-dialysable nitrogen and gain in November–February was 0·867 over all animals, which indicated that 75% of the variation in weight gain of the individual steers in this period was accounted for statistically by the excretion of non-dialysable nitrogen.

The extent to which differences in excretion of non-dialysable nitrogen are responsible for differences in gain is not so easily determined. The simplest explana-

tion for the strong correlation between non-dialysable nitrogen excretion and gain is that the excretion of this component is highly correlated with intake. This would mean that the animals with the biggest intake of pasture nitrogen excreted the most non-dialysable nitrogen, and were the biggest animals (Table 4). The relationship between intake and excretion of nitrogen cannot be defined precisely in grazing animals, because the intake of nitrogen cannot be measured. If non-dialysable nitrogen excretion was significant to gain solely by virtue of its correlation with intake, then the percentages derived for the statistical relationship would represent minimum proportions of variation in gain accounted for by intake.

In this experiment at least, more than half of the variation in gain in the grazing steers was associated with variation in non-dialysable nitrogen excretion. This fairly simple measurement may prove useful in selecting superior performers in a group of young beef cattle. The repeatability of non-dialysable nitrogen excretion of individual steers between the two periods was 0·890 ($P < 0·01$). However, its eventual use as a selection criterion will depend on its heritability, which has not yet been determined.

(*b*) *Breed Differences in Gain and Excretion*

If non-dialysable nitrogen excretion is considered a reasonable indicator of intake, the superior growth rate of the crossbred steers during November–February (2·38 lb/day), compared with the British steers (2·05 lb/day), is due in large part to a greater pasture intake. Thus the mean excretion of non-dialysable nitrogen by the crossbreds during this period was 40·04 g/day, and by the British steers 32·58 g. However, from the results it cannot be determined whether the crossbreds grow faster because of inherent breed differences in eating ability, or whether they eat more because they become progressively larger as a result of some other factor, e.g. superior digestive capacity. The present data give little indication of inherent breed differences in excretion of non-dialysable nitrogen, the regression coefficients derived for the three breeds from interrelationships between faecal nitrogen component excretion, gain, and body weight not differing significantly in any instance. If, as discussed above, intake and non-dialysable nitrogen excretion are highly correlated, then this would imply that there are no striking, categorical breed differences in intake.

There was a tendency, just significant, for the Zebu crossbreds to gain less in the July–October period and more in the November–February period than the British steers. The average excretion of non-dialysable nitrogen by the British steers, as a percentage of that for the crossbred steers, was 91·0% in July–October and 81·4% in November–February (from Table 3). This implies a relatively greater intake by the crossbred steers in November–February, or a relatively smaller intake in July–October, or both, compared with the British steers. From the results of climate chamber experiments (Johnson, Ragsdale, and Yeck 1958) a relatively greater reduction of food consumption by non-Zebu steers might be anticipated under conditions of heat stress. However, Lampkin and Quarterman (1962) found that, though the diurnal grazing pattern of British and Zebu cattle differed in the coastal plains of east Africa, the actual time spent grazing (and by inference the pasture consumed) did not show a breed difference.

(c) *Relationship between Dialysable Faecal Nitrogen and Gain*

It has been found that about half the difference in weight gain between British and Zebu crossbred steers is related to differences in excretion of dialysable nitrogen (Ashton 1962). This proportion between breeds also applies within breeds. Further work has shown that the difference in excretion is probably due to differences in absorption of the end-products of nitrogen digestion (unpublished observations).

The difference in absorption was revealed by feeding steers the same amount of ration. It is not known how dialysable nitrogen varies with amount and composition of feed or with body weight, and so the grazing animals of this study cannot be compared critically with regard to dialysable nitrogen excretion. However, during the four collections in the period July–October the steers were excreting fairly small quantities of non-dialysable nitrogen, and it is reasonable to assume that during this period the intake of nitrogen of the steers was more nearly similar than in November–February. The correlation between dialysable nitrogen and gain in July–October was $-0{\cdot}722$ ($P < 0{\cdot}01$) over all steers, confirming the relationship found previously.

In November–February, however, the correlation coefficient was $0{\cdot}544$ ($P < 0{\cdot}05$). One possible explanation of this is that the animals with the biggest intake in summer are also the most efficient digestors. Whether or not this is so can only be answered by estimating digestive efficiency and grazing intake on the same group of animals.

V. Acknowledgments

I am indebted to Mr. H. G. Turner for statistical advice and criticism of the manuscript, to Mr. J. F. Kennedy, Officer-in-Charge, National Cattle Breeding Station, "Belmont", Rockhampton, for providing the steers and faecal collection facilities and to his staff for making the faecal collections, and to Misses Cruden, Perkins, and Steer for technical assistance.

VI. References

Ashton, G. C. (1962).—*J. Agric. Sci.* **58**: 333.

Forbes, R. M. (1950).—*J. Anim. Sci.* **9**: 231.

Hutchinson, K. J. (1958).—*Aust. J. Agric. Res.* **9**: 508.

Ivins, J. D., *Ed.* (1959).—"Measurement of Grassland Productivity." (Butterworths Sci. Publ.: London.)

Johnson, H. D., Ragsdale, A. C., and Yeck, R. G. (1958).—Res. Bull. Mo. Agric. Exp. Sta. No. 683.

Kennedy, J. F., and Turner, H. G. (1959).—C.S.I.R.O. Aust. Div. Anim. Hlth. Prod., Divl. Rep. No. 8 (Ser. S.W.-3).

Lambourne, L. J. (1958).—Proc. Aust. Agrostology Conf., Armidale, 1958. Pap. No. 51.

Lampkin, G. H., and Quarterman, J. (1962).—*J. Agric. Sci.* **59**: 119.

Lancaster, R. J. (1954).—*N.Z. J. Sci. Tech.* **36**: 15.

Milford, R. (1957).—*Aust. J. Agric. Res.* **8**: 359.

Raymond, W. F., Kemp, C. D., Kemp, A. W., and Harris, C. E. (1954).—*J. Brit. Grassl. Soc.* **9**: 69.

SECTION "E": ANALYTICAL METHODS

Paper No.	Title	Describes: -
32	Method for the determination of propamidine and M & B 1270 (dibromopropamidine by means of pentacyano-ammonioferrate. (1950)	-first sensitive colorimetric procedure for blood.
33	A colorimetric determination of dihydrostreptomycin. (1953)	-a method for estimation of dihydrostreptomycin in admixture with other antibiotics.
34	Paper electrophoresis of streptomycin. (1953)	-application of the then relatively new technique of paper electrophoresis to antibiotics in fermentation media.
35	Actinomycetes metabolism: α phenylmannosidase of Streptomyces grisens. (1954)	-properties of mannosido-streptomycinase.
36	Isotope-dilution technique for determining benzylpenicillin in fermentation liquors. (1955)	-first use of ^{14}C labelled benzyl-penicillin for its specific assay in biological materials.
37	Griseofulvin and dimethylformamide. (1955)	-solubility properties of griseofulvin; (a patent was subsequently issued on this discovery).
38	Determination of griseofulvin in fermentation samples. Pt. I. Spectrophotometric assay. (1956)	-first non-biological assay for the antibiotic griseofulvin. (This assay is now widely used.)
38a	Appendix: Seven point correction procedure.	-an extension of the Morton-Stubbs triangulation correction, which has gained acceptance in general spectrophotometric techniques.
39	Determination of griseofulvin in fermentation samples. Pt. II. Isotope dilutions assay. (1956)	-a more accurate alternative to the method described in paper No. 38.
40	A method of correcting for irrelevant absorption in ultraviolet spectrophotometric analysis. (1956)	-a more detailed mathematical treatment of 38a.
41	Isotope dilution assay of antibiotics in fermentation liquors. (1956)	-a theoretical treatment of isotope-dilution assays.

Reprinted from THE ANALYST, *the Journal of the Society of Public Analysts and Other Analytical Chemists, August,* 1950, *Vol.* 75, *No.* 893, *pp.* 425–430

Method for the Determination of Propamidine and M & B 1270 (Di-Bromo-Propamidine) by means of Pentacyanoammonioferrate

BY HILDA TROUGHT, G. C. ASHTON AND R. G. BAKER

SYNOPSIS—A method for the determination of Propamidine and M & B 1270 by means of pentacyanoammonioferrate has been demonstrated, and its application to the estimation in blood serum has been given preliminary trials, the range of blood level so far found being between 2 and 10 mg. per 100 ml. calculated as free base. The method is capable of being carried out in an ordinarily equipped hospital laboratory.

THE estimation of the aromatic diamidine type of chemotherapeutic agents has been extensively investigated in the last few years owing to the need of a method for the control of their clinical applications. Fuller[1] described a method of determination based on the reaction of aromatic diamidines with a glyoxal - bisulphite compound, using boric acid as a buffer. Jackson, Kuhl and Irvin[2] elaborated Fuller's glyoxal - bisulphite method by the addition of benzaldehyde to the aromatic diamidine, the reaction being developed in alkaline solution. Under suitable experimental conditions, fluorescent glyoxalidone derivatives are formed and can be quantitatively determined on a fluorimeter.

The method described is based on the addition of Fearon's pentacyanoammonioferrate reagent,[3] referred to in this paper as PCAF, to an aqueous solution of the aromatic diamidine; the compound chosen for the development of the method was propamidine isethionate because of its reasonable solubility in water. Under controlled experimental conditions, the addition of PCAF results in the formation of a yellow to orange coloration which varies quantitatively with the concentration of the diamidine in solution, provided the concentration is not too high. At high concentrations a coloured complex is thrown out of solution and can be filtered off and dried as an apparently homogeneous orange-coloured substance, from which the original base may be recovered quantitatively.

Fearon suggests, as a possible formula for an amidine + PCAF compound,

$$(Fe^{III}(CN)_5NH_2C(NH).R)'''$$

From a somewhat limited analysis of the propamidine + PCAF compound, it is suggested that it is formed from one molecule of PCAF + one molecule of diamidine. Further analysis would have to be made, however, before this chemical structure is definitely established.

PREPARATION OF CALIBRATION GRAPHS FOR PROPAMIDINE ISETHIONATE

REAGENTS—

Pentacyanoammonioferrate—A 1 per cent. solution, diluted to 1 in 10 immediately before use.

Propamidine isethionate—Aqueous solutions of 1·0, 0·10 and 0·05 per cent. concentration.

Two calibration curves were prepared by adding calculated quantities of a standard 1 per cent. propamidine isethionate solution to pre-determined volumes of water contained in test tubes in order to bring the combined volumes to 5 or 3 ml. To the prepared dilution, 1 ml. of the diluted PCAF solution was then added, bringing the final volumes to 6 and 4 ml. respectively. The colour was allowed to develop for 20 to 30 minutes, after which time it underwent no further observable deepening. The readings were taken on a photo-electric colorimeter, using a blue-green filter, OB2, the solutions being placed in test tubes specially matched for use in the colorimeter.

The reagents alone gave a reading of 2 units, and this blank has been subtracted from the figures, given in Tables I and II.

TABLE I

DATA FOR CALIBRATION GRAPH FOR THE DETERMINATION OF PROPAMIDINE ISETHIONATE IN A 6-ML. TEST SOLUTION

Propamidine isethionate, mg.	2	1·5	1·0	0·5	0·2	0·15	0·10	0·05	0·025	0·012	0·006
Galvanometer reading* ..	79	60	41	26	11	8	5	3	2·5	2	1

* A reagent blank of two divisions has been subtracted.

TABLE II

DATA FOR CALIBRATION GRAPH FOR THE DETERMINATION OF PROPAMIDINE ISETHIONATE IN A 4-ML. TEST SOLUTION

Propamidine isethionate, mg. ..	1·5	1·0	0·5	0·2	0·10	0·05
Galvanometer reading*	108	71	36	15	7	4

* A reagent blank of two divisions has been subtracted.

Calibration graphs of galvanometer readings on a logarithmic scale plotted against milligrams of propamidine isethionate proved to be straight lines passing through the origin.

I. Galvanometer reading 26 ≡ 0·5 mg. of propamidine isethionate in 6·0 ml.
II. Galvanometer reading 36 ≡ 0·5 mg. of propamidine isethionate in 4·0 ml.

From Table I it can be seen that 12 μg. of propamidine isethionate, corresponding to 6 μg. of the free base, in 6·0 ml. of solution, can be determined; the lowest figure was discarded, as it was outside the limits of experimental error.

Two of the authors (G. C. A. and R. G. B.) independently checked the validity of the principle of the above experiments by preparing calibration curves for a 1·833 per cent. solution of the propamidine isethionate, which corresponds to a theoretical 1 per cent. solution of the free base, and subsequent dilutions. Using a Beckmann spectrophotometer for the observations, quantities down to 5 μg. could be detected.

The virtual agreement of these independent results is the basis for the establishment of the PCAF method of estimating propamidine. The method can be extended to the estimation of M & B 1270 and M & B 1314, for which analogous calibration curves can be prepared.

ESTIMATION OF AMIDINES IN BIOLOGICAL FLUIDS

The application of the method to the estimation of propamidine, M & B 1270 and M & B 1314 in blood serum was next investigated. A modification of Jackson's method[2] for the removal of protein by dialysed iron followed by centrifugation was used to obtain, with comparative regularity, a clear supernatant fluid. The diamidine content of this fluid was then estimated by means of the PCAF reagent. A test with a serum blank must be made at the same time and the value subtracted from the reading for the unknown amount of diamidine.

Although Fearon's reagent gives orange colours with urea, creatine, guanidines and other substances occurring in blood, at the concentrations usually present the colours produced appear relatively negligible, and remain so over a wide range of concentrations. For example, a solution of urea corresponding to 500 mg. per 100 ml. gave no significant galvanometer reading.

A series of serum recovery experiments was carried out by adding known quantities of the diamidine to measured quantities of stock serum and finding the percentages that can be quantitatively recovered.

PROCEDURE—

Place 2·5 ml. of serum in an Erlenmeyer flask and add 7·0 ml. of water. Heat over a small flame until the solution is definitely beginning to boil. Stop heating, and add 2·0 ml. of dialysed iron dropwise from a pipette, swirling the contents of the flask after the addition of each drop. Return the flask to the flame for about 1 to 2 minutes, and continue the swirling.

It can usually be seen at this stage whether the fluid is going to be clear. Transfer the contents of the flask to a centrifuge tube, and centrifuge. The supernatant fluid should be perfectly clear; if it is not, it can sometimes be cleared by putting it back into the flask and re-boiling gently, but the boiling should not be prolonged so as to alter the volume

significantly. If the solution still does not clear, it should be discarded and the procedure recommenced.

Between 5 and 6 ml. of clear supernatant fluid should be obtained in this way.

COLORIMETRIC DETERMINATION OF DIAMIDINE—

Place 2 ml. of the supernatant fluid, 1 ml. of distilled water and 1 ml. of PCAF reagent into a test tube, allow to stand of half an hour, and then read the absorption on the photo-electric colorimeter. This value is the serum blank of the stock serum. Table III gives details of serum recovery experiments carried out by this method.

TABLE III

Known quantities of diamidine added to 2·5 ml. of stock sera

PROPAMIDINE ISETHIONATE								
Added, mg.	20	10	5	3	2·5	2·0	2·0	1·0
Determined, mg.	23·0	11·5	5·2	2·64	2·3	2·07	1·9	1·2
	20·4	10·0						
DI-BROM-PROPAMIDINE								
Added, mg.	2·0	1·0	1·0	0·4	0·1	0·1		
Determined, mg.	1·90	0·96	1·12	0·52	0·07	0·12		

EXPERIMENTS *in vivo*

A series of experiments *in vivo* were carried out on patients undergoing treatment with M & B 1270 either by intravenous injection or by irrigation of drainage tubes in surgical procedures; the results of these experiments are given in Table IV.

TABLE IV

	Nov. 5	Nov. 9	Nov. 12	Nov. 22
Patient 1	9·0 mg.	5·0 mg.	6·5 mg.	2·4 mg.
	Dec. 21		Jan. 5	Jan. 10
Patient 2	10 mg.		6·8 mg.	3·7 mg.
Patient 3	13 mg.			
Patient 4	6·3 mg.			
	1st day	5th day		
Patient 5	2·8 mg.	2·8 mg.		

NOTES—

Patient 1—Daily intravenous injections of 100 mg. of M & B 1270 started about 5 days before the first specimen of blood was taken for quantitative analysis.

Patient 2—Daily irrigation of drainage tube from a subphrenic abscess by 1 g. of M & B 1270 in 20 ml. of saline.

Patients 3 *and* 4—Irrigation of drainage tube with M & B 1270.

All the percentages are calculated as free base. For each patient a serum blank must be determined, preferably before medication with the drug begins; if this is not practicable, the blank should be determined not less than 10 days after discontinuation of the drug.

Interference from the simultaneous use of the M & B "Sulpha" triad appears to be negligible at the concentrations commonly used. A minimum percentage of 240 mg. of sulphathiazole or 100 mg. of sulphamerazine or sulphadiazine is necessary before any colour is given with the PCAF reagent.

The accuracy of low level determinations of diamidine in blood serum could be increased by the use of 2·0-ml. cells in the photo-electric colorimeter, although this involves the determination of calibration curves in 2·0-ml. cells.

Occasionally anomalous results are obtained, and it is possible that some interference, at present unknown, is caused by the phenomenon of *cis* and *trans* isomerism closely associated with some of the diamidines.

The authors thank the Clinical Staff at Dudley Road Hospital for their co-operation and collaboration in allowing access to patients who were undergoing post-operative treatment

with M & B 1270; Dr. Whitelaw, Director of the laboratories; Dr. W. R. Thrower, M.R.C.P., and Dr. H. Campbell, of May & Baker Ltd., who initiated the Diamidine Research at Dudley Road Hospital, without whose helpful criticism and encouragement the work would not have reached this stage.

References

1. Fuller, A. T., *Biochem. J.*, 1945, **39**, 99.
2. Jackson, D. P., Kuhl, W. J., and Irvin, J. L., *J. Biol. Chem.*, 1947, **167**, 377.
3. Fearon, W. R., *Analyst*, 1946, **71**, 562.

Biochemical Department
Dudley Road Hospital
Birmingham, 18

First submitted, *July*, 1949
Amended, *January*, 1950

(33)

A Colorimetric Determination of Dihydrostreptomycin

BY

G. C. ASHTON, M. C. FOSTER and M. FATHERLEY

Reprinted from THE ANALYST, *the Journal of the Society of Public Analysts and Other Analytical Chemists, October*, 1953, *Vol.* 78, *No.* 931, *pp.* 581-585

A Colorimetric Determination of Dihydrostreptomycin

By G. C. ASHTON, M. C. FOSTER and M. FATHERLEY

A colorimetric method for the determination of dihydrostreptomycin is presented. It is based on a reaction between guanido materials and diacetyl, alkali and α-naphthol. The colour formed is not subject to interference at high salt concentrations and can be used in the routine analysis of factory samples. Penicillin and its compounds do not interfere with the reaction, so the method can be applied to the determination of dihydrostreptomycin in mixtures with penicillin.

The standard error of analyses made by the method is ±1·3 per cent., which compares favourably with that found in microbiological assay.

Several chemical methods are available for determining dihydrostreptomycin. In general they are either tedious or subject to interference from substances other than dihydrostreptomycin in the samples submitted for assay. We required a simple method for determining dihydrostreptomycin in a variety of samples ranging from process control samples to samples of the pure antibiotic alone and in admixture with other antibiotics.

Oxidimetric, spectrophotometric and colorimetric methods have been published for the determination of dihydrostreptomycin. The oxidimetric procedures depend on the oxidation of dihydrostreptomycin by sodium metaperiodate[1] or periodic acid.[2,3] Formaldehyde is produced, and, after distillation under carefully controlled conditions, the formaldehyde is determined with chromotropic acid. The necessity for distillation was overcome by Vail and Bricker,[3] who removed the interfering periodic and iodic acids by lead acetate precipitation and developed the colour of chromotropic acid with formaldehyde in the clear centrifugate. Methods based on periodate oxidation suffer from the disadvantage that streptomycin produces different amounts of formaldehyde under different conditions. In our experience such careful control of this procedure is required as to make it inconvenient for routine use.

A method based on the spectrophotometric absorption of dihydrostreptomycin at 265 mμ after acid hydrolysis was suggested by Hiscox[4] and is suitable for pure dihydrostreptomycin, but it cannot be applied to mixtures of antibiotics without the use of appropriate correction factors, which reduce its precision.

As the dihydrostreptomycin and streptomycin molecules are identical except that the aldehyde grouping of the streptose moiety is hydrogenated in dihydrostreptomycin and is therefore not reactive, colorimetric methods for streptomycin based on the streptidine or N-methylglucosamine moieties are potentially applicable to the determination of dihydrostreptomycin. Hence, the streptidine assay developed by Sullivan and Hilmer[5] for streptomycin has been applied to dihydrostreptomycin by Monastero.[6] The streptomycin assay described by Scudi, Boxer and Jelinek[7] is based on the Elson - Morgan reaction for glucosamine and, similarly, can be used for dihydrostreptomycin estimation on the nearly pure substance, but dihydrostreptomycin gives only one twenty-fourth of the colour given by streptomycin with the same technique and reagents.

Our objective being to develop for dihydrostreptomycin an assay that could be applied routinely to a variety of samples, as mentioned above, we investigated a number of reactions dependent on the streptidine moiety of the molecule. Such a reaction is not specific for dihydrostreptomycin; it is also given by streptomycin and mannosidostreptomycin. Streptomycin and dihydrostreptomycin occur together at certain stages during dihydrostreptomycin manufacture, and in assaying these samples for dihydrostreptomycin by a streptidine assay it is necessary to allow for the streptomycin present. This can be done conveniently with the ferric maltol assay,[8] which determines streptomycin but not dihydrostreptomycin. Mannosidostreptomycin is not usually present in the streptomycin used for the manufacture of dihydrostreptomycin, but if any were present it would be estimated by the ferric maltol assay. It is unusual to find streptomycin and dihydrostreptomycin associated in samples of mixed antibiotics. Hence, for practical purposes a streptidine assay can be used as a measure of dihydrostreptomycin content in mixed antibiotic samples.

Experimental

Several possible methods based on reactions of the guanidine groups of the streptidine moiety were examined. The method of Monastero[6] with the oxidised nitroprusside reagent

of Weber[9] is subject to interference by salts. A method for aromatic amidines[10] is applicable to dihydrostreptomycin, but was found also to be subject to salt interference.

We also investigated extensively the Sakaguchi reaction,[11] using combinations of α-naphthol or 8-hydroxyquinoline with hypochlorite[12] or hypobromite.[13] We found the resulting colours to be unstable and could not obtain reproducible results with any combination of the reagents.

A recent paper by Halliday[14] described a spray reagent for streptomycin chromatograms. The reaction involved is attributed to Voges-Proskauer, and the spray reagent used is based on Barritt's[15] procedure for guanidine materials, involving the use of diacetyl, α-naphthol and potassium hydroxide. This reaction was finally selected as the basis of our method.

Halliday[14] said that the diacetyl colour with streptomycin developed slowly and faded after reaching a maximum optical density. We have confirmed this, although the fading rate and time at which the maximum optical density occurs depend on the relative concentrations of the three reagents.

ESTABLISHMENT OF OPTIMUM CONCENTRATIONS—

The original instructions[15] for applying the diacetyl reaction to guanidine materials were as follows. To 1 ml of guanidine solution add 1 ml of a 0·1 per cent. solution of diacetyl in water, 2·25 ml of water, 0·5 ml of 5 per cent. ethanolic α-naphthol and 0·25 ml of 40 per cent. alcoholic potassium hydroxide. These amounts of reagent proved suitable for aqueous solutions of dihydrostreptomycin, but from solutions containing much salt the salts were precipitated owing to the high alcohol concentration of the final solution. This was prevented by preparing the caustic potash solution in water rather than in methanol, but on addition of the α-naphthol solution this reagent was itself precipitated. If, however, the potash was added before the α-naphthol, no precipitation occurred. Changes in reagent volumes were then made, so that 2 ml of sample were diluted with 15 ml of water and 1 ml of each of the three reagents was added.

As already mentioned, the time of maximum colour development and the amount of colour formed depend on the relative concentrations of the three reagents. Experiments with different combinations of 0·1, 0·2 or 0·4 per cent. diacetyl, 10, 20 or 40 per cent. aqueous potassium hydroxide and 2·5, 5 or 10 per cent. alcoholic α-naphthol were carried out. The optical density of each solution after standing for 30, 60 and 90 minutes was measured. From these results it appeared that 0·4 per cent. diacetyl, 20 per cent. potassium hydroxide and 10 per cent. α-naphthol solutions gave maximum colour formation. This combination of reagents gave a colour that remained at its maximum value for 10 to 15 minutes.

WAVELENGTHS OF MAXIMUM ABSORPTION—

The wavelengths at which the solutions from the first experiment absorbed most strongly were determined; $\lambda_{max.}$ ranged from 505 mμ to 530 mμ. With the combination of reagents finally adopted, the maximum absorption was at 525 mμ.

EFFECTS OF TIME AND TEMPERATURE—

A solution of dihydrostreptomycin in water was treated with 0·4 per cent. diacetyl, 20 per cent. potassium hydroxide and 10 per cent. α-naphthol solutions at 21°, 23°, 25° or 27° C. Aliquots were removed at 5-minute intervals and the optical densities measured at 525 mμ against water. The results are shown in Fig. 1. Both time and temperature have a marked effect on the rate at which the colour develops, but the final colour is always maximal after about 40 minutes and is fully developed between 23° and 27° C. It is, therefore, recommended that the temperature at which colour development is carried out should be not less than 23° C, although satisfactory results can be obtained at lower temperatures. Further, it is necessary to measure the developed colours at a fixed time after adding the reagents; we have found 40 minutes to be suitable.

BLANK VALUE OF THE REAGENTS—

The reagents themselves give rise to a coloured solution, the intensity of the colour increasing with time (see Fig. 1). Therefore, if a series of samples is being assayed at timed intervals it is not practicable to use the same reagent blank value. Rather than develop a separate reagent blank for each sample, we find it convenient to measure the developed

sample colour against water, and subtract from this measurement the optical density value of a separately developed and similarly timed reagent blank measured against water.

CALIBRATION GRAPH—

An apparently linear calibration graph is obtained with dihydrostreptomycin solutions containing up to 400 units per ml when the final coloured solution is measured in a 1-cm cell at 525 mμ against water. This line passes through a point on the ordinate corresponding to the blank value of the reagent. Calibration is also linear with a Spekker absorptiometer and No. 604 filters.

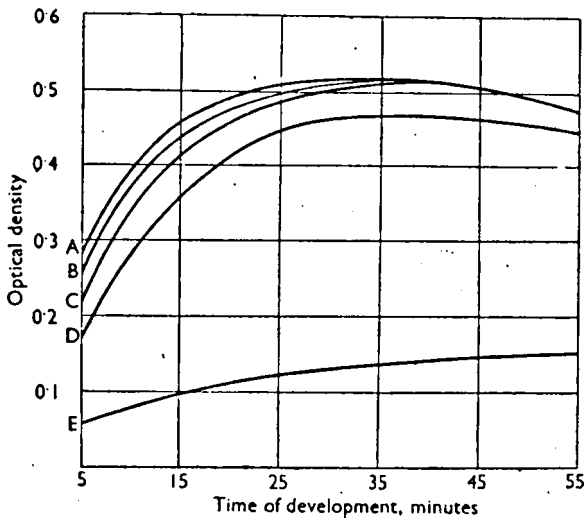


Fig. 1. Effect of time and temperature on development of colour between dihydrostreptomycin and the diacetyl-α-naphthol reagent. Curve A, 27° C; curve B, 25° C; curve C, 23° C; curve D, 21° C; curve E, reagent blank

STABILITY OF REAGENTS—

From experiments with combinations of reagents of different ages, it appears that the diacetyl and potassium hydroxide solutions are stable for at least 2 weeks. The slope of the graph relating optical density to concentration increases slightly as the α-naphthol solution ages, an effect not solely due to an increased blank value, but good results have been obtained with α-naphthol solutions 2 weeks old.

EFFECT OF CONTAMINANTS ON COLOUR—

The effect on the diacetyl determination of substances that might be encountered in pharmaceutical preparations of mixed antibiotics, or during the production of dihydrostreptomycin, was determined. The substances chosen were added to a solution containing 250 units of dihydrostreptomycin per ml.

No colour suppression or intensification was obvious when the dihydrostreptomycin solutions contained 10 per cent. w/v of anhydrous sodium sulphate, 5 per cent. w/v of anhydrous sodium citrate, 1 per cent. w/v of calcium chloride ($CaCl_2.6H_2O$), 0·2 per cent. w/v of sodium benzylpenicillin, 0·03 per cent. w/v of procaine penicillin or 0·03 per cent. w/v of penethamate hydriodide.

The effect on the diacetyl reaction of substances other than those mentioned has not been investigated. Caution should be exercised in applying the method to pharmaceutical preparations containing vegetable oils, ointment bases or unknown excipients.

The diacetyl reaction is also given by streptomycin and mannosidostreptomycin, streptidine, arginine and other guanido derivatives.

Method

Reagents—

Diacetyl solution—A 0·4 per cent. w/v solution of diacetyl in distilled water.

Potassium hydroxide solution—A 20 per cent. w/v solution of potassium hydroxide in distilled water.

α-Naphthol solution—A 10 per cent. w/v solution of α-naphthol in absolute ethanol.

Procedure—

Either dissolve the sample in distilled water or dilute liquid samples to give a solution containing between 100 and 400 units of dihydrostreptomycin per ml (0·1 to 0·4 mg per ml of dihydrostreptomycin base).

Transfer 2 ml of the sample solution to a 6-inch × 1-inch test tube, add 15 ml of water and then 1 ml of diacetyl solution, 1 ml of potassium hydroxide solution and 1 ml of α-naphthol solution, in that order. Mix the contents of the tube by inversion after each addition. Start timing when the diacetyl solution is added. Precisely 40 minutes later determine the optical density at 525 mμ in a 1-cm cell against water. Alternatively, measure the optical density on an absorptiometer with a suitable filter, *e.g.*, a Spekker absorptiometer with No. 604 filters.

Prepare a standard graph from suitable dilutions of a standard dihydrostreptomycin solution, using the same procedure for colour development as described for the sample. Determine the potency of the diluted sample solution from the standard graph. A standard graph should be prepared for each determination to allow for differences in room temperature, reagents, and so on.

Results and discussion

Precision—

An examination of the results from replicate determinations on different samples at one concentration level showed the apparent standard error of a determination to be ±0·6 per cent. However, observations on replicate determinations at several concentrations of one sample showed the standard error to be greater than this. It seems likely, therefore, that the graph of optical density and concentration has a slight curvature over the range 0 to 400 units per ml.

To determine the precision attainable over the recommended range, three different dihydrostreptomycin samples were dissolved in water to give solutions containing approximately 400 to 450 units per ml. Each solution was then diluted accurately with water to give solutions of concentrations 75, 50 and 25 per cent. of the original solutions. The three original solutions and their nine dilutions were assayed in duplicate by the method described. The potencies of the twelve solutions were determined from a standard graph prepared from a standard dihydrostreptomycin material.

The results of this experiment are shown in Table I.

Table I

Potencies of dihydrostreptomycin solutions at four concentrations

Dilution level, %	Sample A	Sample B	Sample C	Level average
25	423·1	426·1	420·1	427·02
	431·9	429·0	431·9	
50	424·5	417·2	409·9	415·25
	418·7	414·3	406·9	
75	420·1	417·2	410·3	416·55
	424·1	419·2	408·4	
100	421·6	426·7	414·3	420·02
	419·4	423·8	414·3	
Sample average	422·93	421·69	414·50	419·71

An examination of the results showed that the major part of the error is due to the non-agreement between results from different levels on any one sample, caused by slight curvature of the graph of optical density and concentration. The error between duplicate determinations at one level on one sample is considerably less than the major error due to curvature.

For routine purposes, however, this curvature error is insufficient to warrant special precautions (such as restricting values to the linear portion of the graph or making allowances for the curvature). The standard error of a pair of duplicate determinations, including the error due to curvature, is about ± 1·3 per cent.

COMPARISON WITH BIO-ASSAY—

A number of dihydrostreptomycin sulphate samples and various dihydrostreptomycin solutions were assayed both microbiologically by the *Klebsiella pneumoniae* plate assay and chemically by the method described. The results are shown in Table II.

TABLE II

COMPARISON BETWEEN PROPOSED METHOD AND MICROBIOLOGICAL ASSAY

Solid samples		Solutions	
By diacetyl method, units per mg	By microbiological method, units per mg	By diacetyl method, units per ml	By microbiological method, units per ml
765	750	343	365
778	780	341	340
770	780	354	350
765	767	318	340
		326	335
		326	330

There are no significant differences between the chemical and microbiological results.

APPLICATION TO STREPTOMYCIN—

It is clear that the method described is also applicable to streptomycin and gives results similar to those by the ferric maltol method,[8] both with simple streptomycin solutions and with mixed antibiotic samples. The method cannot, however, be used for the determination of the streptomycin in fermentation broths, as it gives a measure of the total guanidine-reacting material present in the broth. We believe the chief value of the method lies in its application to dihydrostreptomycin, for which the more specific ferric maltol method cannot be used.

We are indebted to Mr. J. P. R. Tootill not only for carrying out the statistical analysis of the results, but also for useful criticism and helpful suggestions in the planning of experiments during the development of the method. We wish also to thank Mr. K. A. Lees for carrying out the microbiological assays.

Note added in proof.—After this paper had been accepted for publication, a paper appeared describing the application of the Voges - Proskauer reaction to the quantitative assay of streptomycin (Szafiv, J. J., and Bennett, E. O., *Science*, 1953, **117**, 717).

REFERENCES

1. Garlock, A. E., jun., and Grove, D. C., *J. Clin. Invest.*, 1949, **28**, 843.
2. Colon, A., Herpich, G. E., Johl, R. G., Neuss, J. D., and Frediani, H. A., *J. Amer. Pharm. Ass., Sci. Ed.*, 1950, **39**, 335.
3. Vail, W. A., and Bricker, C. E., *Anal. Chem.*, 1952, **24**, 975.
4. Hiscox, D. J., *Ibid.*, 1951, **23**, 923.
5. Sullivan, M. X., and Hilmer, P., Abstract of the 109th Meeting of the American Chemical Society, April, 1946, p. 4B.
6. Monastero, F., *J. Amer. Pharm. Ass., Sci. Ed.*, 1952, **41**, 322.
7. Scudi, J. V., Boxer, G. E., and Jelinek, V. C., *Science*, 1946, **104**, 486.
8. Boxer, G. E., Jelinek, V. C., and Leghorn, P. M., *J. Biol. Chem.*, 1947, **169**, 153.
9. Weber, C. J., *Ibid.*, 1928, **78**, 465.
10. Trought, H., Ashton, G. C., and Baker, R. G., *Analyst*, 1950, **75**, 437.
11. Sakaguchi, S., *J. Biochem., Tokyo*, 1925, **5**, 25.
12. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, 1945, **159**, 185.
13. Vincent, D., and Brygoo, P., *Bull. Soc. Chim. Biol.*, 1946, **28**, 43.
14. Halliday, W. J., *Nature*, 1952, **169**, 335.
15. Barritt, M. M., *J. Path. Bact.*, 1936, **42**, 441.

GLAXO LABORATORIES LTD.
SEFTON PARK
STOKE POGES, BUCKS.

February 24th, 1953

(34)

(*Reprinted from Nature, Vol.* 172, *p.* 958, *November* 21, 1953)

Paper Electrophoresis of Streptomycins

MANNOSIDOSTREPTOMYCIN in some streptomycin preparations is conveniently detected by the paper chromatographic procedure of Winston and Eigen[1]. In the presence of high concentrations of salts such as in ion-exchange column eluates, or in fermentation broths, this procedure is not applicable: attempts to desalt such solutions, by mixed-bed ion-exchange, electrolytic de-ionization or solvent extraction, have proved unsuccessful. We have found it possible to separate streptomycin, mannosidostreptomycin and allied substances by paper electrophoresis, having developed (independently) a technique similar to that used for new antibiotic broths by Hosoya *et al.*[2], and King and Doery[3].

The apparatus is simple and consists of (*a*) a pair of electrode vessels ('Pyrex' dishes, 17 cm. × 27 cm. × 5·5 cm.), in each of which is situated a pure graphite electrode (20 cm. × 2·5 cm. × 0·5 cm.) with a platinum lead; (*b*) the support and cover for the filter paper (a pair of planar sheets of plate glass 28 cm. × 58·5 cm.), arranged horizontally on levelling screws between the electrode vessels. A cooling system was found to be unnecessary with a power pack providing 0–100 m.amp. at 300 volts.

Several grades of filter paper were examined; the most regular spots and highest loadings were obtained with Whatman No. 31 (thick). The paper is cut to size (24 cm. × 70 cm.), and the origin line is drawn 7·5 cm. from one end.

The solutions to be examined are applied to the origin line from a micrometer pipette, and the spots are allowed to dry. The paper is wetted evenly with *p*H 5·0 buffer (700 ml. 0·2 *M* sodium acetate plus 300 ml. of 0·2 *N* acetic acid) to within 0·5 cm. of each side of the origin line. The paper is blotted between blotting paper with a rubber roller and placed in position on the bottom sheet of levelled plate-glass, and the upper glass sheet is laid on top. About 300 ml. of buffer solution is added to each electrode vessel. The end of the paper farthest from the origin line is dipped into the cathode vessel, and the buffer is allowed to rise up the paper until the origin line is wetted and electrolyte continuity thus established. The end of the paper nearest the origin line is then dipped into the anode vessel, and the current is switched on. Sixteen hours later (overnight) the current is turned off and the paper is removed and dried, ready for spraying or laying on an agar plate seeded with a suitable organism[1].

We have examined a number of spray reagents; the following modified reagents are all suitable for detecting small quantities of streptomycin on paper.

(*a*) A diacetyl spray[4], which indicates streptomycin by means of its guanido groupings. The reagent (equal volumes of 0·1 per cent v/v aqueous diacetyl, 20 per cent w/v aqueous potassium hydroxide and 2·5 per cent w/v alcoholic α-naphthol mixed, in the order given, immediately before use) is sprayed on the air-dried paper. A magenta colour develops within a few minutes at room temperature if 5 μgm. or more of streptomycin equivalent is present.

(*b*) A naphthoresorcinol spray originally described[5] for the detection of carbohydrates, with a sensitivity for streptomycin similar to that of (*a*). The dry paper is sprayed evenly with the reagent (0·5 gm. of naphthoresorcinol dissolved in 225 ml. of ethanol and acidified with 25 ml. of concentrated orthophosphoric acid) and dried in an air oven at 100° C. for a minute or two until the background is faintly pink. The streptomycin spots are strongly fluorescent when viewed in ultra-violet light. In much higher concentrations the spots are visible in daylight, that from mannosidostreptomycin being pink and from streptomycin greyish.

(*c*) A spray reagent suggested by Partridge[6] for the detection of glucosamine, based on the Elson–Morgan reaction. The dry paper is sprayed with solution *A* (5 ml. of 50 per cent w/v alcoholic potassium hydroxide mixed, just before use, with 100 ml. of 1 per cent v/v acetyl acetone in redistilled *n*-butanol) and is heated in an oven at 100° C. for five minutes. After cooling, it is resprayed with solution *B* (0·5 gm. of *p*-dimethylaminobenzaldehyde dissolved in 15 ml. of ethanol, acidified with 7 ml. of concentrated hydrochloric acid and added to 90 ml. of redistilled *n*-butanol). Pink spots appear after reheating in the 100° oven for two or three minutes. This spray reacts with the N-methylglucosamine portion of the molecule. The reaction is somewhat less sensitive than that of (*a*) or (*b*).

A single paper may be used for sprays (*a*) and (*c*).

Table 1

Substance	Mobility ($\mu \times 10^{-5}$)
Streptomycin	22·5
Mannosidostreptomycin	19·5
Streptothricin	24·0
Streptidine	24·9
Streptamine	6·3

The paper is first sprayed with the diacetyl reagent and the resultant spots are outlined before treatment with the Elson–Morgan sprays. It is necessary to use a separate paper for the naphthoresorcinol spray.

Table 1 shows average values for mobility, μ, calculated from the formula $\mu = dl/vt$, where d is the distance in cm. from the origin to the centre of the spot, v is the average of initial and final voltages applied and observed, l is the length of the paper in cm., and t is the time of migration in seconds. (These values are not corrected for electro-endosmosis; the effect of this was tested with glucose and found to be negligible with the buffer and paper recommended.)

The technical assistance of Miss I. Thompson is gratefully acknowledged.

M. C. Foster
G. C. Ashton

Glaxo Laboratories, Ltd.,
Stoke Poges,
Buckinghamshire.
Aug. 7.

[1] Winsten, W. A., and Eigen, E., *J. Amer. Chem. Soc.*, **70**, 3333 (1948).
[2] Hosoya, S., Soeda, M., Komatsu, N., Hara, N., Sonoda, Y., and Arai, R., *J. Antibiot. Jap.*, **4**, 314 (1951).
[3] King, N. K., and Doery, H. M., *Nature*, **171**, 878 (1953).
[4] Halliday, W. J., *Nature*, **169**, 335 (1952).
[5] Partridge, S. M., *Biochem. J.*, **42**, 238 (1948).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

[FROM THE BIOCHEMICAL JOURNAL, VOL. 57, No. 1, pp. 93–98, 1954]
 PRINTED IN GREAT BRITAIN

Actinomycete Metabolism: α-Phenylmannosidase of *Streptomyces griseus*

BY D. J. D. HOCKENHULL, G. C. ASHTON, K. H. FANTES AND BELINDA K. WHITEHEAD
Glaxo Laboratories, Ltd., Sefton Park, Stoke Poges, Bucks

(*Received* 23 *September* 1953)

Langlykke & Perlman (1950) have reported that *Streptomyces griseus* forms an enzyme capable of converting mannosidostreptomycin into the aglycone, streptomycin, and have described the dependence of its activity upon oxidation potential and pH value and its inhibition by heavy metals. During the course of our work it was found that synthetic phenyl α-D-mannoside was also split by the organism. Accordingly we have used this synthetic substrate to provide such general information on α-mannosidase activity as might later be used in work on the natural substrate. Although in this work the term 'mannosidase', which is used for convenience, implies a simple hydrolysis of mannosidostreptomycin or α-phenylmannoside into mannose and streptomycin or phenol, we do not exclude the possibility that the enzymic cleavage of either substrate is achieved by a more complex mechanism.

METHODS AND MATERIALS

Organism. Streptomyces griseus mutant (Dulaney, Z38) was maintained and grown on soya bean medium, as in example I of the patent describing it (Dulaney, 1951). Growth was always carried out at 28·5° on a rotary shaker (160 rev./min.) in quantities of 40 ml. medium in 250 ml. conical flasks.

Determination of enzyme activity. The substrate was phenyl α-D-mannoside, prepared by the method of Helferich & Winkler (1933) with the modification that deacetylation was carried out as by Zemplen & Kunz (1923). The determinations were based on measurement of the amount of phenol liberated.

In our early experiments we removed the phenol by distillation, using a method similar to that described by Volterra (1942) but the pH was adjusted to 6·4 with 0·1 M-KH_2PO_4, and CO_2 was also bubbled through the boiling liquid to prevent frothing. The phenol content of the distillate was determined colorimetrically (Folin & Ciocalteu, 1927). This method not only proved tedious, but also needed large samples, and simpler ones were tried. Extraction with chlorinated solvents (cf. Ellinger, Ruchhoft & Lishka, 1951) was difficult on account of persistent emulsion formation. A search was then made for reagents for estimating phenol which were not subject to interference by broth constituents. The procedure of Gottlieb & Marsh (1946), based on colour development with 4-aminoantipyrin, was found suitable in sensitivity, specificity and stability of colour formed. It was found convenient to clear the samples by addition of ethanol, which did not interfere with the test. Other methods of colorimetric estimation were rejected, that of Folin & Ciocalteu (1927) because of inconsistent blank values, that with diazotized sulphanilic acid (Schmidt, 1949) because of the instability of the colour and that involving the use of halogenated quinone chlorimides to form coloured indophenols (Singer & Stern, 1951) because of the slow rate of attaining maximum colour.

Enzyme activity was defined as being unity when 1 μm-mole of phenol is liberated per ml. in 30 min. at the temperature of incubation. The activity varies with temperature and unless otherwise stated all determinations recorded here were carried out at 28·5°. The enzyme is sensitive to aeration and therefore a standard set of conditions is laid down for the operation of the test, as below. The reagents used were: *A*, glycine buffer, pH 10·4, prepared by mixing 54 vol. of an aqueous solution containing 4·51 g. glycine and 3·48 g. NaCl in 600 ml. and 46 vol. of 0·1 N-NaOH; this is adjusted to pH 10·4 with the appropriate constituent solutions; *B*, 1% (w/v) aqueous 4-aminoantipyrin; *C*, 3% (w/v) aqueous potassium ferricyanide A.R.; *D*, 1·0% (w/v) aqueous α-phenylmannoside.

Incubation unit. A 6 × 1 in. boiling tube with a 'B24' socket to fit a gas wash bottle head (MF 28/3, Quickfit and Quartz, Ltd. London), whose inlet tube reaches to within 8 mm. of the bottom was immersed in a temperature-controlled water bath and connected in series to other units and an air supply delivering 700–900 ml./min. A vibrator-type aquarium aerator was found most suitable for a constant-rate air supply.

Enzyme activity. 15 ml. of enzyme solution (e.g. culture fluid) were transferred into the incubation unit, which was allowed to equilibrate to the required temperature for 5 min. To this was added 1 ml. of *D*; the aeration train was then connected and incubation was carried out for exactly 30 min. Immediately after this, 14 ml. of 96% ethanol (industrial methylated spirits) were added with good mixing and the solution was then filtered through a Whatman no. 42 fluted filter.

To 1 ml. of filtrate were added 10 ml. *A*, 1 ml. *B* and 3 ml. *C*, with good mixing. The colour developed was measured in a Spekker Absorptiometer (Ilford no. 605 filters; $\lambda_{max.}$, 545 mμ.) against an enzyme blank consisting of 1 ml. of filtrate from a mixture of 15 ml. of enzyme solution and 15 ml. of 96% ethanol. The readings must be taken within 10–40 min. after the reagent additions.

Standard calibration graphs were constructed by adding 1 ml. *B* and 3 ml. *C* to the following mixtures:

glycine buffer *A* (ml.)	10	9	8	7	6
5×10^{-6} M phenol (ml.)	1	2	3	4	5
to give units of enzyme activity	100	200	300	400	500

The developed colours were read against a water blank. The phenol solutions were checked by bromimetric or iodimetric analysis.

The method was checked to show the reproducibility of the phenol assay by determination of added phenol (Table 1), and the reproducibility of enzyme activity for the same broth samples (Table 2). Agreement was found to be excellent. We also investigated the effect of storage at −10° on the enzyme in whole cultures and found that after 4 days no deterioration in activity had occurred.

It has also been found that the requisite aeration may be obtained by shaking 15 ml. of enzyme preparation in a 250 ml. conical flask on a rotary shaker, as described above, at 28·5°. This method was used for many of the determinations quoted below, as it was useful for carrying out a number of investigations at the same time, when comparative rather than absolute values were important. The units of activity, which differ little from those obtained in tubes (units (*T*)/ml.), will be given as units (*S*)/ml.

Whole cultures were used as enzyme sources. Very little work has yet been carried out on the purification of the enzyme.

RESULTS

Effect of time and substrate concentration

Under the conditions of aeration, temperature and pH described above, the amount of phenol liberated by the enzyme increased linearly with time (Fig. 1). In experiments in which enzyme action was allowed to continue beyond a point at which the amount of liberated phenol was about 200 μg./ml., some slowing down was observed. This was first thought to be due to an inhibitory effect of phenol, but was found not to be so, as the addition of phenol (200 μg./ml.) caused no retardation of enzyme activity. On the other hand, mannose (380 μg./ml.) did cause some inhibition.

For accurate enzyme estimations it is necessary to work on the portion of the time curve that is linear, i.e. up to about 2 μmole of liberated phenol/ml. broth.

Determination of the Michaelis constant

Though the Michaelis constant for a pure enzyme and the substrate concentration at which the enzyme shows one half its maximum activity in culture broth are not necessarily identical, the constant is still of some use in characterizing the enzyme. A number of determinations were made with 10 ml. portions of broth to which was added 1 ml. of aqueous substrate solution containing sufficient α-phenylmannoside to give concentrations of 136, 55, 27 and 11 μg./ml. of incubation mixture. Incubation with shaking was carried out for 10 min. and the liberated phenol estimated in the usual way. A typical set of figures is shown in Table 3.

In the range, 136–55 μg./ml., the liberation of phenol was, within experimental error, the same. Below this level, the hydrolysis of α-phenylmannoside was complete at the end of the incubation

Table 1. *Recovery of added phenol from non-incubated whole cultures*

For details of the estimation of phenol see text. *A* and *B* represent duplicate determinations on the same fortified broth culture sample; *a* and *b* represent duplicate colorimetric determinations on the same ethanolic filtrates.

Sample	Added phenol (μg./ml.)	Phenol recovered (μg./ml.)		Recovery (%)	
		a	*b*	*a*	*b*
1*A*	16·9	17·9	17·5	105·5	103·5
1*B*	16·9	16·7	16·7	98·8	98·8
2*A*	33·1	32·1	32·5	97·1	98·2
2*B*	33·1	32·9	32·9	99·4	99·4
3*A*	53·7	52·5	53·3	97·8	99·3
3*B*	53·7	54·2	53·8	101·0	100·1
4*A*	63·7	61·9	61·9	97·2	97·2
4*B*	63·7	63·7	63·7	100·0	100·0
		Average recovery		99·6	99·6

Table 2. *Reproducibility of enzyme activity determinations under standard conditions*

For details of the estimation and definition of enzyme activity see text. Standard deviation of individual determination, 1·4%.

Culture sample	Enzyme activity (units (*T*)/ml.)		
A	294	289	289
B	187	188	195
C	564	552	554
D	952	962	953
E	277	276	276
F	366	365	365

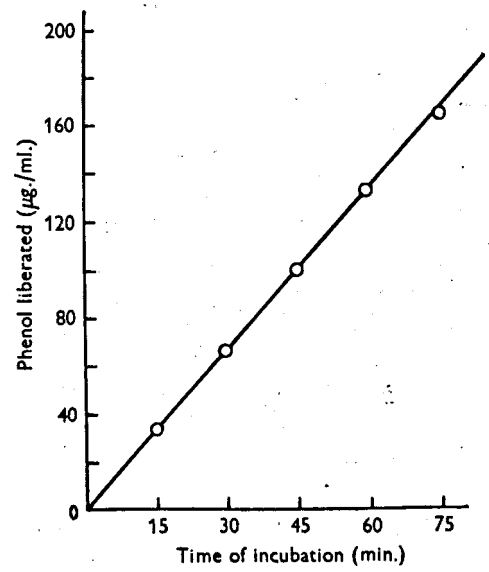


Fig. 1. Relationship between time of incubation and liberation of phenol from α-phenylmannoside by whole cultures of *Streptomyces griseus*. For conditions see text.

Table 3. *The effect of substrate concentration on enzyme activity*

10 ml. portions of culture broth were incubated with varying amounts of α-phenylmannoside in 1 ml. water. For further details see text.

Substrate concentration (μg./ml.)	Phenol liberated (μg./ml.) In experiment	Phenol liberated (μg./ml.) Theoretically possible
136	14·2	50·0
55	13·7	20·0
27	10·3	10·0
11	4·4	4·0

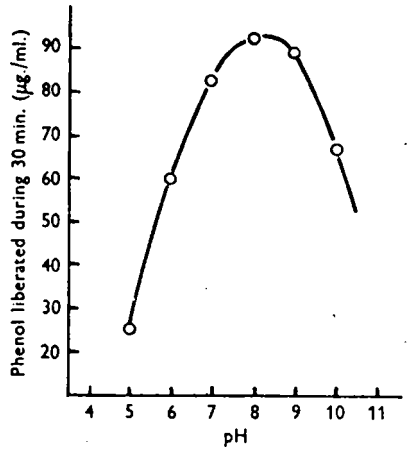


Fig. 2. The effect of pH on the hydrolysis of α-phenylmannoside by whole cultures of *Streptomyces griseus*. For conditions see text.

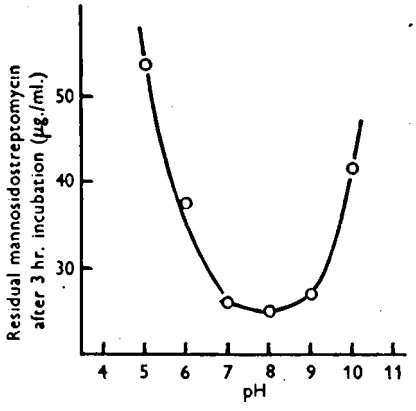


Fig. 3. The effect of pH on the hydrolysis of mannosidostreptomycin. For conditions see text.

period and the liberated phenol gives no indication of the reaction velocities. In order to measure the initial velocities, shorter incubation periods would have to be chosen in such a way that the liberated phenol could be measured before total hydrolysis occurs, but the amount of liberated phenol would then be too small for accurate estimation. All that can be deduced from the present experiments is that a Michaelis constant in excess of 10 μg./ml., i.e. approx. 4×10^{-5} M, would not be consistent with the experimental findings.

Effect of hydrogen-ion concentration

Samples of a standard 6-day culture were adjusted to various pH values with N-HCl or N-NaOH and brought to equal volumes by small compensating additions of water. The activity (units (*S*)/ml.) was determined for each under otherwise standard conditions. Fig. 2 indicates that optimum activity was shown at about pH 8·0, but that there was less than a 10 % fall from this value at either pH 7·0 or 9·0. This showed that, as active cultures without exception had pH values between 7·0 and 9·0, there was only need to adjust the pH value of the enzyme system in the most exacting experiments. As a rule, therefore, this was not done. Beyond these limits activity declined rapidly to 30 % of maximum at pH 5·0 and 70 % of maximum at pH 10·0.

A similar experiment with mannosidostreptomycin as substrate (Fig. 3) indicated that the optimum of the culture for cleavage of this substrate was again about pH 8. In this experiment 15 ml. portions of culture broth (containing a negligible amount of mannosidostreptomycin) were adjusted to various pH levels as in the above experiment. Solid mannosidostreptomycin hydrochloride was added to give a concentration of 100 μg./ml. The mixtures were incubated with shaking for 3 hr. (trial experiments had shown that the cleavage of mannosidostreptomycin was much slower than that of α-phenylmannoside). Residual mannosidostreptomycin was then estimated by the method of Emery & Walker (1949).

Effect of aeration upon enzyme activity

Enzyme—substrate mixtures were made as for the standard shake-flask test, but were distributed in various quantities in smaller (100 ml.) conical flasks, in order to vary the degree of aeration. Incubation with shaking of 15, 45 and 75 ml. portions of digest for 15 min. gave enzyme activities of 740, 600 and 220 units (*S*)/ml., respectively.

A more precise experiment was carried out under the conditions of the tube test, which was set up in the standard way except that the rate of passage of air was varied (Fig. 4). It is clear that the rate of aeration must be fixed; for the standard test, as mentioned above, it was set at 700–800 ml./min. The apparent decrease in enzyme activity with very high aeration is due to loss of phenol from the incubation unit, as was proved by passing the exit air through alkali and detecting phenol in the alkali.

Inhibition by anaerobiosis was reversible, shown by incubating broth anaerobically in presence of substrate and subsequently under aerobic conditions. Three 10 ml. portions of an enzyme-substrate digest were treated in the following way. The first was incubated anaerobically for 30 min., the second aerobically for 30 min. and the third portion aerobically and then anaerobically for the same length of time; 12, 49 and 56 μg./ml. phenol were liberated in the respective digests. This experiment was repeated and the same effect obtained after 16 hr. of anaerobiosis.

A similar dependence on aeration has been found when mannosidostreptomycin was used as substrate. In a typical experiment 20, 30, 40 and 50 ml. of culture broth to which 620 μg./ml. mannosidostreptomycin had been added were placed into 50 ml. conical flasks, which were then plugged with cotton wool. A fifth flask was filled completely with a similar mixture and closed with a rubber bung. Mannosidostreptomycin was determined after incubating with shaking for 3 hr.; 90, 85, 100, 320 and 550 μg./ml. of mannosidostreptomycin were found in the respective flasks.

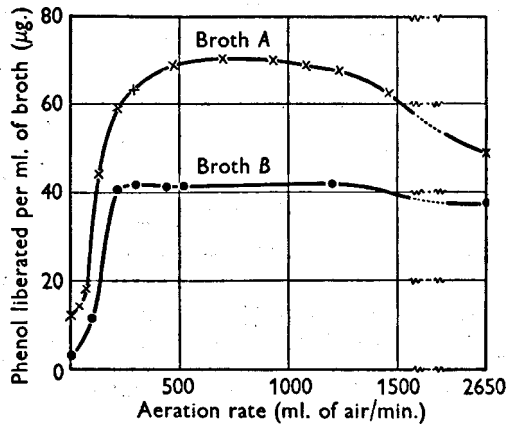


Fig. 4. Showing the effect of aeration on the α-phenylmannosidase activities of two broths (*A* and *B*) of *Streptomyces griseus*. For conditions see text.

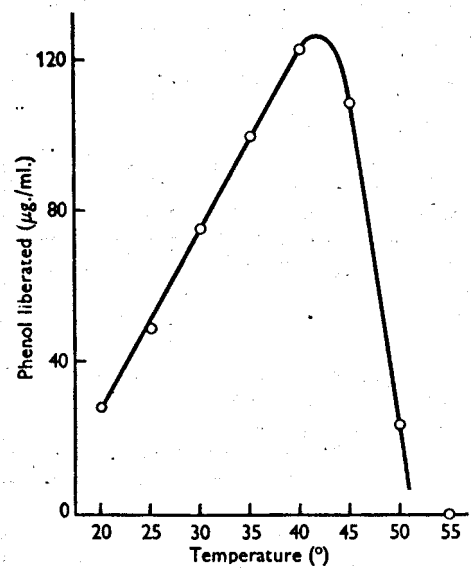


Fig. 5. Effect of temperature on the hydrolysis of α-phenylmannoside. For conditions see text.

Effect of temperature upon enzyme action

Under standard shaking conditions the optimum temperature for enzyme action was found to be about 40°. The enzyme appears to be very heat labile and is completely inactivated at 55° (Fig. 5).

The effect of phosphate on enzyme activity

In order to decide whether the cleavage of α-phenylmannoside was hydrolytic or phosphorolytic, incubation was carried out on the shaker in the presence of 0·02 M sodium phosphate of the same pH as the broth. To the control broth the same amount of sodium (as sodium chloride) was added, to eliminate any effect due to sodium. The results are shown in Fig. 6. A similar experiment with 0·05 M phosphate again showed that phosphate did not increase the rate of hydrolysis; it is therefore unlikely that α-mannoside is split phosphorolytically. (The concentration of inorganic phosphate of a normal 6-day old culture filtrate at the time of determination of enzyme activity was about 0·0003 M.)

Effect of inhibitors

Inhibition due to sugars. A number of sugars were added to enzyme preparations at 0·05 M concentration and their effect on the liberation of phenol from α-phenylmannoside over a period of 30 min. (with shaking) was ascertained. The results from a number of experiments on different broths are shown in Table 4.

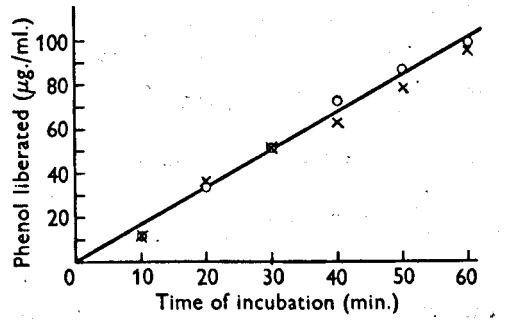


Fig. 6. Effect of added phosphate on rate of hydrolysis of α-phenylmannoside. ×—×, Added 0·02 M phosphate; O—O, control. For conditions see text.

Effect of metal ions. The enzyme activity was reduced only by relatively high concentrations of Cu^{2+} and Fe^{2+} (as sulphates). In a number of culture broths these metals at a concentration of 0·001 M had no effect on enzyme activity, whereas a concentration of 0·01 M invariably produced strong enzyme inhibition. In some experiments, however, there was also some inhibition with 0·001 M metal salts. K^+, Mg^{2+}, Ca^{2+} and Ba^{2+} (all 0·01 M) had no effect on enzyme activity. The enzyme activities in these experiments were determined after incubation on the shaker.

Effect of sulphite, iodoacetate, arsenate and cyanide. Solutions of the sodium salts of these substances, adjusted to the pH of the culture broth with sodium hydroxide or hydrochloric acid, were added to give a concentration of 0·01 M. Incubation with shaking for 30 min. in the presence of substrate showed that sulphite and cyanide caused 62 and 90 % inhibition, respectively, arsenate stimulated enzyme activity by 40 % and iodoacetate had no effect. (These figures represent average values from several experiments.)

Table 4. *Effect of carbohydrates on enzyme activity*

Duplicate figures for the inhibition by individual sugars were obtained from separate digests, not from samples of the same digest. For other details see text.

Sugar (0·05 M)	Approx. % inhibition
Glucose	5, 2, 7
Fructose	7, 7, 0
D-Arabinose	7, 13
D-Ribose	2, 6
D-Xylose	11
Rhamnose	0, 0
Sucrose	13, 3, 2, 12
Mannitol	4, 0
α-Methylglucoside	9, 20, 12
Maltose	66, 81
Cellobiose	80, 84
Mannose	87, 87, 80, 79
α-Methylmannoside	98, 93, 96, 96

Correlation of mannosidostreptomycin level with appearance of phenyl α-D-mannosidase activity

In a number of experimental cultures the amount of mannosidostreptomycin was determined by the method of Emery & Walker (1949) and compared with the α-mannosidase activity over a number of days. It was found that high mannosidase activity was paralleled by a low level of mannosidostreptomycin. When there was no fall in mannosidostreptomycin level, little or no enzyme activity was manifest (Table 5).

DISCUSSION

The sensitivity of the enzyme to aeration conditions is noteworthy. A similar phenomenon for hydrolytic enzymes was described recently (Yamagita & Nishi, 1952), when it was shown that proteolytic hydrolysis by trypsin or chymotrypsin and the autocatalytic conversion of trypsinogen into trypsin were inhibited under anaerobic conditions. Since cell lysis starts soon after the disappearance of glucose from the medium and is followed by the appearance of α-mannosidase activity, it would appear possible that the enzyme might be intracellularly present earlier in the fermentation, and that lysis might activate it by bringing it into contact with a higher oxygen tension.

The affinity of the enzyme for α-phenylmannoside was found to be higher than for its natural substrate mannosidostreptomycin. The Michaelis constant for the latter has been found in our laboratories to be $1·72 \times 10^{-3}$ M (P. B. Dickenson & J. S. Hughes, personal communication). Owing to the difficulty of determining phenol at the necessary low levels, we were not able to estimate the Michaelis constant for the synthetic substrate. It seems, however, to be appreciably lower than that found for the natural substrate. Moreover, the rate of hydrolysis of mannosidostreptomycin was only

Table 5. *Relation between mannosidostreptomycin content and α-phenylmannosidase activity of cultures*

% *M*, mannosidostreptomycin as molar % of total streptomycin. *E*, α-phenylmannosidase activity (units (S)/ml.; in *A*, *B*, *C*, *D* and units (T)/ml.; in *E*, *F*, *G*, *H*, *I*).

	Duration of fermentation									
	Day 3		Day 4		Day 5		Day 6		Day 7	
Culture sample	% *M*	*E*	% *M*	*E*	% *M*	*E*	% *M*	*E*	% *M*	*E*
A	—	—	46	0	50	0	60	40	—	0
B	—	—	39	0	8	1490	6	940	5	600
C	51	0	49	0	46	54	36	20	—	—
D	34	0	41	0	5	1930	8	1750	6	1120
E	—	—	—	—	33	0	32	0	29	0
F	31	0	33	35	43	45	29	175	—	—
G	31	0	30	35	25	205	3	995	—	—
H	56	0	32	245	8	1605	7	1235	—	—
I	40	0	43	0	19	905	7	340	—	—

about one-sixth of that of α-phenylmannoside. A similar finding was made by Cohn & Monod (1951), who showed that the affinity of an *Escherichia coli* β-galactosidase for synthetic *o*-nitrophenyl β-galactoside was greater than for lactose.

When breakdown to streptomycin occurs, the pH of a culture broth is usually just on the alkaline side, i.e. at the optimum pH for the action of the enzyme on either substrate. This optimum is in marked contrast to the optimum pH for the action of the α-mannosidases of almond emulsin and lucerne seeds (pH 3·3–5·5; Hill, 1934) or coffee emulsin (pH 4·5–5·5; Helferich & Vorsatz, 1935).

In view of the lack of effect of inorganic phosphate on the rate of hydrolysis of phenyl α-D-mannoside, which implies non-phosphorolytic breakdown, it is strange that arsenate should so markedly increase the reaction velocity.

Only four of the sugars tested, mannose, α-methylmannoside, maltose and cellobiose, were inhibitory. The first two probably acted as competitive inhibitors. It is not clear why the last two, an α-glucoside and a β-glucoside, should inhibit. It is possible that an irreversible enzyme-glucoside complex is formed. α-Phenylglucoside was tried as an enzyme substrate but was not hydrolysed.

The inhibition of the enzyme by the heavy metals copper and iron was presumably of a general nature. Although some changes in the activity of similar microbial glucosidases have been reported with K^+, Ca^{2+}, Mg^{2+} and Ba^{2+}, such an effect was not demonstrated on our enzyme. The failure to observe it may have been due to the use of a cell suspension instead of a purified enzyme.

Of general inhibitors, only sulphite, cyanide and iodoacetate were examined. The two former inhibited strongly, whereas iodoacetate had no effect. It appears, therefore, that the enzymic action of mannosidostreptomycinase is unlikely to depend on the presence of SH groups.

The late appearance in normal cultures of an enzyme releasing mannose from mannosidostreptomycin (mannose, as the phenylhydrazone, was isolated from such cultures by Perlman & Langlykke, 1948) makes it likely that the latter is a carbohydrate storage material for the organism (cf. Frey-Wyssling, 1942) and that release of carbohydrate is brought about when the glucose is depleted. The action of the mould on phenyl α-D-mannoside parallels this and would tend to support the hypothesis that cleavage of both substrates is effected by the same enzyme. The similarity of the pH activity curves found with the two substrates, the dependence of enzyme action for each substrate on aeration and the close correlation between mannosidostreptomycin level and α-mannosidase activity all suggest such an identity.

SUMMARY

1. It has been demonstrated that cultures of *Streptomyces griseus* possess an enzyme capable of liberating phenol from phenyl α-D-mannoside.

2. The optimum pH for the activity of the enzyme is approximately 8·0.

3. The enzyme is very sensitive to conditions of aeration. Decreased aeration causes a marked decrease of enzyme activity.

4. Mannose, methyl α-D-mannoside, maltose and cellobiose strongly inhibit the enzyme. A number of other sugars have little or no effect.

5. Ferrous and cupric ions have some inhibitory effect on the enzyme. K^+, Ca^{2+}, Mg^{2+} and Ba^{2+} have no effect.

6. The rate of enzymic cleavage of substrate is not influenced by inorganic phosphate. Arsenate increases the rate.

7. Cyanide and sulphite inhibit the enzyme, iodoacetate has no effect.

8. The Michaelis constant of the enzyme with respect to phenyl α-D-mannoside is less than 10^{-4} M.

9. It is probable that the same enzyme is responsible for the conversion of mannosidostreptomycin into streptomycin and the hydrolysis of phenyl α-D-mannoside.

The authors wish to acknowledge the valuable technical assistance of Mr I. McCalla, Mr A. Rees and Miss P. Jackson.

REFERENCES

Cohn, M. & Monod, J. (1951). *Biochim. biophys. Acta*, **7**, 153.
Dulaney, E. L. (1951). U.S. Patent, 2571693.
Ellinger, M. B., Ruchhoft, C. C. & Lishka, R. J. (1951). *Analyt. Chem.* **23**, 1783.
Emery, W. B. & Walker, A. D. (1949). *Analyst*, **74**, 455.
Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
Frey-Wyssling, A. (1942). *Naturwissenschaften*, **33**, 500.
Gottlieb, S. & Marsh, P. B. (1946). *Ind. Engng Chem.* (*Anal.*) **18**, 16.
Helferich, B. & Vorsatz, F. (1935). *Hoppe-Seyl. Z.* **237**, 254.
Helferich, B. & Winkler, S. (1933). *Ber. dtsch. chem. Ges.* **66**, 1556.
Hill, K. (1934). *Ber. sächs. Ges.* (*Akad.*) *Wiss.* **86**, 115.
Langlykke, A. F. & Perlman, D. (1950). U.S. Patent, 2493489.
Perlman, D. & Langlykke, A. F. (1948). *J. Amer. chem. Soc.* **70**, 3968.
Schmidt, E. G. (1949). *J. biol. Chem.* **179**, 211.
Singer, A. J. & Stern, E. E. (1951). *Analyt. Chem.* **23**, 1511.
Volterra, M. (1942). *Amer. J. clin. Path.* **12**, 525, 580.
Yamagita, T. & Nishi, A. (1952). *Symposium on Enzyme Chem.* (*Japan*), **7**, 98.
Zemplen, G. & Kunz, A. (1923). *Ber. dtsch. chem. Ges.* **56**, 1705.

(36)

Isotope-dilution Technique for Determining Benzylpenicillin in Fermentation Liquors

BY

G. C. ASHTON and M. C. FOSTER

Reprinted from THE ANALYST, *the Journal of the Society for Analytical Chemistry, February*, 1955, *Vol.* 80, *No.* 947, *pp.* 123-132

Isotope-dilution Technique for Determining Benzylpenicillin in Fermentation Liquors

By G. C. ASHTON and M. C. FOSTER

The chemical assay of benzylpenicillin in fermentation liquors must be based upon the determination of its phenylacetyl grouping, a procedure complicated by the presence of other phenylacetyl derivatives in the sample. An isotope-dilution assay has been devised: it is based on the use of carbon-14 as tracer in benzylpenicillin, is satisfactorily accurate and can have, if due attention is paid to detail, a standard error of no more than ±1 per cent.

The determination of benzylpenicillin in fermentation liquors is complicated by the presence of other penicillins and biologically inactive degradation products, as well as phenylacetic acid and its derivatives used as precursors.

Separation of these substances can be achieved by paper chromatography, and a quantitative estimate of benzylpenicillin as a percentage of the other penicillins then made by the bio-autographic technique.[1] Alternatively, the penicillin-hydroxamic acid derivatives can be chromatographically separated for colorimetric estimation of the percentage of benzylpenicillin after elution from the paper.[2] Although under suitable conditions these methods can be fairly precise, it is necessary to refer the result obtained to some estimate of total penicillins in the broth.

Any chemical assay of benzylpenicillin must be based upon the determination of its phenylacetyl grouping, its only distinction from other penicillins. This, however, involves its separation from other substances in the broth containing phenylacetyl groupings, in itself an analytical problem of some magnitude. Boxer and Everett[3] have described a procedure dependent upon differential solvent extraction of the penicillins coupled with an estimate

of the non-benzylpenicillin phenylacetyl blank. Levy, Fergus and Caldas[4] made use of the N-ethylpiperidinium salts of penicillins as a means of separating the penicillins from the phenylacetyl derivatives. The method gives a result within ± 10 per cent. of the true value.

Another approach to the problem is that of Craig, Tyndall and Senkus,[5] who use ^{13}C-benzylpenicillin in an isotope-dilution procedure involving measurement of the dilution in a mass spectrometer. In a similar manner Trenner and Chase[6] make use of deuterated benzylpenicillin and measure the isotope dilution by infra-red spectrophotometry. Both these procedures are accurate, but their precision is limited by the precision of the measuring instrument, or, with the infra-red measurement, by the precision achieved in dissolving a small quantity of phenylacetic acid in a small volume of volatile solvent.

The procedure described here makes use of ^{14}C-benzylpenicillin and measurement of the isotope dilution with an end-window Geiger - Müller tube and associated electronic equipment. With this equipment the precision is, in practice, limited only by the counting time.

Experimental

The basis of the carbon-14 isotope-dilution assay for penicillin is as follows. A weighed quantity of radioactive benzylpenicillin ("label")* is added to a known volume of the broth sample submitted for assay. The penicillins are then extracted, and the phenylacetic acid (derived from the original benzylpenicillin in the broth and diluted with the added benzylpenicillin from the label) is isolated in pure form. The radioactivity of the isolated phenylacetic acid is compared with the radioactivity of the phenylacetic acid derived solely from the label, and the dilution of the isotope by the inactive penicillin from the broth is computed. The benzylpenicillin content of the original broth can then be calculated from the volume of the broth taken and the weight of label used.

Isotope-dilution assays are free from the usual limitations associated with conventional methods of analysis; thus, the isolation of the measured derivative (in this assay phenylacetic acid) does not have to be quantitative. The accuracy of the assay is dependent mainly on the purity of the isolated derivative and the capabilities of the physical equipment used for measuring the isotope dilution. However, unless large quantities of sample and label are to be used it is necessary to have a reasonable efficiency at each stage to obtain sufficient phenylacetic acid for the physical measurements required. Thus the experiments during the development of the assay were chiefly associated with the establishment of a procedure giving reasonable yields of pure phenylacetic acid from a manageable quantity of broth.

In practice the procedure consists of four steps for broth samples—

(*a*) A weighed quantity of radioactive benzylpenicillin label is added to a known volume of filtered broth and most of the penicillin then present is extracted through solvent into phosphate buffer.

(*b*) The phosphate buffer extract may contain phenylacetic acid derived from precursors added to the fermentation, and thus the extracted penicillin is separated by crystallising a suitable penicillin derivative.

(*c*) The crystalline derivative is hydrolysed with alkali and the phenylacetic acid (originating from the benzylpenicillin in the broth and from the added label) is crystallised and recrystallised.

(*d*) The pure phenylacetic acid is subjected to radioactivity measurement, and compared with the radioactivity of the phenylacetic acid similarly derived from the label.

Solvent extraction of penicillins from broth—

Various solvent-extraction schemes were investigated. In these laboratories we use ether for quantitative extraction of small volumes of broth for polarimetric assay of total penicillins. However, with the broth volumes involved in the isotope assay (500 ml) this procedure is unwieldy and gives insufficient concentration at the buffer extract stage. Amyl and butyl acetates have been used as solvents for penicillin extraction,[4,7] but in our experience difficulty in separating emulsions is frequently encountered with these solvents. Boxer

* In describing work with material that has been "labelled" by means of an isotope, there appears to be some inconsistency of terminology. It is often only clear from the context whether the word "tracer" is being applied to the isotope itself or to the labelled material. In an attempt to standardise terminology, we have throughout this paper used the word "tracer" to signify the carbon-14 incorporated in the synthetic material required for the isotope-dilution assay. The sample of material so labelled we shall describe simply as the "label."

and Everett[3] use chloroform as an extraction solvent, and recommend anhydrous sodium sulphate as a means of breaking any emulsion produced. This procedure works well, and a single chloroform extraction gives consistent yields to the buffer stage of about 75 per cent. of the penicillin present in the sample.

A sample of penicillin medium was filtered and divided into three portions, and aliquots of a solid containing 98·3 per cent. of benzylpenicillin were added to each portion to give artificial "broths" of potency 1145, 2050 and 3285 units of benzylpenicillin per ml. Each "broth" was treated in triplicate by taking the indicated volume, adding a quantity of penicillin label, adjusting to pH 2·0 to 2·2 with phosphoric acid and extracting with twice the volume of chloroform. The chloroform extract was re-extracted with three quantities of pH 7·0 phosphate buffer. The quantity of penicillin in the combined buffer extract was determined by polarimetric rotation, by using a previously determined conversion factor. The extraction efficiencies are shown in Table I.

TABLE I

EFFICIENCY OF BROTH EXTRACTION WITH CHLOROFORM

	Broth A			Broth B			Broth C		
Volume of broth, ml	500			250			150		
Volume of chloroform, ml	900			500			300		
Volume of buffer, ml	50 + 35 + 35			35 + 25 + 25			25 + 15 + 10		
Replicate number	1	2	3	1	2	3	1	2	3
Weight of penicillin in broth sample, mg	349	349	349	312	312	312	300	300	300
Weight of added label, mg*	285	286	288	289	286	286	285	289	286
Weight of penicillin in buffer, mg	480	492	483	477	466	476	437	428	436
Extraction, per cent.	75·8	77·4	76·0	79·2	77·9	79·7	76·1	72·5	74·3

* As sodium benzylpenicillin.

Fermentation broths gave similar results, duplicate extractions on 300-ml aliquots of a typical broth giving 75·7 and 79·8 per cent. yields.

PREPARATION OF CRYSTALLINE DERIVATIVE—

Two penicillin derivatives have been used for assay purposes, the *iso*propyletherate complex as made by Trenner[5,8] and the N-ethylpiperidinium salt as made by Sheehan, Mader and Cram.[9] Although it is considered that both of these derivatives contain only benzylpenicillin when prepared from penicillin samples containing at least 90 per cent. of benzylpenicillin, it is now conceded that both derivatives may contain substantial amounts of penicillin other than benzylpenicillin when prepared from broth extracts.[10,4] The object

TABLE II

EFFICIENCY OF *iso*PROPYLETHERATE FORMATION

	Broth A			Broth B			Broth C		
Volume of buffer, ml	120			85			50		
Volume of amyl acetate, ml	10 + 10			8 + 8			5 + 5		
Volume of *iso*propyletherate, ml	100			80			50		
Replicate number	1	2	3	1	2	3	1	2	3
Weight of penicillin in buffer, mg	480	492	483	477	466	476	437	428	436
Yield of complex, mg*	311	329	312	324	280	292	292	301	281
Yield as benzylpenicillin, per cent.	64·8	66·9	64·6	68·0	60·1	61·3	66·9	70·2	64·5

* This represents the weight of *iso*propyletherate complex converted to an equivalent weight of sodium benzylpenicillin.

of preparing a crystalline derivative of penicillin then is primarily to separate extraneous phenylacetyl compounds from benzylpenicillin. Levy, Fergus and Caldas[4] have confirmed that gummy N-ethylpiperidine precipitates contain inert material, although they state that crystalline salts contain "negligible" quantities of inert components. It has been our experience that yellow pigment persists throughout the phenylacetic acid crystallisation stages after hydrolysis of the N-ethylpiperidinium salt, resulting in impure phenylacetic acid crystals.

Crystallisation of the *iso*propyletherate complex of penicillin from buffer extracts yields well-shaped yellowish crystals, which on subsequent hydrolysis and crystallisation give colourless phenylacetic acid crystals. Thus we find it preferable to use the *iso*propyletherate complex as a procedure for separating the penicillin in the buffer extract from non-penicillin material.

It was not found convenient to extract the phosphate buffer concentrate directly with *iso*propyl ether, as rapid crystallisation of the complex frequently occurred during the extraction. It was preferable to extract the phosphate buffer with a small quantity of amyl acetate, dehydrate the amyl acetate extract and then add 5 volumes of *iso*propyl ether. On standing in the refrigerator overnight, crystals of penicillinic acid *iso*propyletherate form in good yield. Table II shows the yields obtained from the phosphate buffer extracts derived as in Table I.

PREPARATION OF PURE PHENYLACETIC ACID—

The solvent-free *iso*propyletherate complex is converted to phenylacetic acid. The use of both sulphuric acid and sodium hydroxide hydrolysis was investigated, purer phenylacetic acid crystals being obtained after alkaline hydrolysis. Experiments showed that 4 to 6 ml of 20 per cent. sodium hydroxide and a hydrolysis time of 1 to 3 hours gave the best yields of phenylacetic acid from a given weight of benzylpenicillin, or *iso*propyletherate complex.

A number of solvents were investigated for extracting the phenylacetic acid. Of these, *cyclo*hexane[6] was found to give the least coloured phenylacetic acid residue on evaporation of the solvent.

The phenylacetic acid residue requires recrystallisation, and redistilled light petroleum, boiling range 40° to 60° C, was found to be satisfactory, giving good crystallisation yields and being removed from the crystals with relative ease.

The efficiency of the hydrolysis and crystallisation stages is given in Table III, which is a continuation of Tables I and II.

TABLE III

EFFICIENCY OF HYDROLYSIS AND CRYSTALLISATION STAGES

	Broth A			Broth B			Broth C		
Replicate number	1	2	3	1	2	3	1	2	3
Weight of complex as sodium benzylpenicillin, mg*	311	329	312	324	280	292	292	301	281
Weight of pure phenylacetic acid, mg ..	92	100	92	104	83	79	81	79	86
Yield from complex to recrystallised acid, per cent.	77·2	80·0	77·8	84·7	77·5	71·1	74·5	68·5	80·5
Over-all yield from original broth sample, per cent.	38·1	41·4	37·9	45·4	36·4	34·7	36·4	35·3	38·5

* See Table II.

TREATMENT OF SOLID SAMPLES—

The benzylpenicillin content of finished products may be determined by the isotope-dilution procedure. Most finished products have been through a stage involving formation of a penicillin derivative during manufacture and are thus free from phenylacetic acid originating from added precursors. The penicillin sample plus label can thus be hydrolysed directly with alkali, and the phenylacetic acid extracted and crystallised.

By this procedure 499·6 mg of label (potassium benzylpenicillin) gave 151·3 mg of recrystallised phenylacetic acid, *i.e.*, 83·9 per cent.: 601·6 mg of label plus sample (sodium benzylpenicillin) gave 170·6 mg of phenylacetic acid, a yield of 76·3 per cent.

ACCURACY AND PRECISION OF THE METHOD

The accuracy of the isotope-dilution assay for penicillin is dependent upon the radiochemical purity of the label (*i.e.*, the only radioactive species in the label must be benzylpenicillin), the purity of the isolated phenylacetic acid samples taken for radioactivity measurements, the accuracy of the weighings and volume measurements involved in the initial dilution of the sample with the label and the accuracy of the counting.

It has been our experience, and also that of Sebek,[7] that only radioactive benzylpenicillin is produced when a radioactive phenylacetic acid derivative is used as precursor. This point

can be checked as described below.[10] The purity of the isolated phenylacetic acid is difficult to assess with the small quantities available. Melting-point determinations are of little use. The best approach is adequate recrystallisation, and the carrying out of duplicate isotope assays on any sample. The two results should fall within the limits calculated from the formula for the precision of the assay (see below). The accuracy of the weighing of the label and the measurement of the sample volume (of a broth) will obviously influence the accuracy of the result. Once again, the best approach is to carry out isotope-dilution assays in duplicate on each sample. The accuracy of the counting is dependent on the accuracy of the planchette preparation, particularly as to the weight on the planchette.

Exactly 20, 30 and 40 mg of radioactive phenylacetic acid sample were plated on 1-sq. cm polythene planchettes. The counting rates were respectively 650, 674 and 708 counts per minute. Thus a weight difference of 1 mg in this instance corresponds to a difference in count of about 0·43 per cent. at the 30-mg level. In this manner it is possible to decide what difference in weight between replicate planchettes is possible without the error from this source exceeding the attained counting precision. The characteristics of the planchettes (diameter, evenness of surface, etc.) and the evenness of packing of the phenylacetic acid on the planchette also influence the accuracy of the count.

The best way to check the accuracy of planchette preparation is to count at least two, preferably three, separately prepared planchettes, and test for consistency of the individual counts by the χ^2 test. The accuracy of counting is also dependent on the proper functioning of the Geiger - Müller tube and associated electronic equipment. This may be checked by counting a suitable radioactive standard each time radioactivity measurements are made.

The precision of the estimate is dependent upon the relationship between the various counts involved in the assay, also upon the ratio between units of benzylpenicillin in the sample taken, and units of benzylpenicillin in the added label.

The conditions for obtaining the "best" precision, *i.e.*, the optimum conditions, can be derived mathematically as follows—

Let x = the ratio $\dfrac{\text{units of benzylpenicillin in sample}}{\text{units of benzylpenicillin in label}}$,

$\dfrac{\sigma(x)}{x}$ = standard error of x as a percentage,

t = degraded label count (counts per minute per unit area for a given weight of pure phenylacetic acid derived by degradation of the label),

m = degraded mixture count (counts per minute per unit area for the same weight of pure phenylacetic acid derived by degradation of the benzylpenicillin isolated from the broth sample after addition of the label),

b = counts per minute for background,

β = ratio b/t,

T = total counting time (degraded label + degraded mixture + background),

p = proportion of time for degraded label count,

q = proportion of time for degraded mixture count, and

$(1-p-q)$ = proportion of time for background count.

Then—

$$x = \frac{t-m}{m-b}$$

and therefore—

$$\frac{\sigma(x)}{x} = \frac{100}{(1-\beta)\sqrt{Tt}} \times \left(\frac{1+x}{x}\right)\sqrt{\frac{1}{p} + \frac{(1+x)(1+\beta x)}{q} + \frac{\beta x^2}{1-p-q}}.$$

It is obvious that T and t should be made as large as practicable if the best precision is to be achieved. The total counting time, T, is a matter of convenience. However, the degraded label count, t, is restricted in practice by the "resolving time" of the Geiger - Müller tube.[11] It is inadvisable to have the degraded label count much above 1500 counts per minute, or the lost counts due to the "dead" time of the tube will cause too low a result to be obtained. With T and t made as large as practicable, the proportional standard error $\sigma(x)/x$ is at a minimum when—

$$p = \frac{1}{3+\sqrt{\beta}},$$

$$q = \frac{2(1+\sqrt{\beta})^2}{(3+\sqrt{\beta})(1+3\sqrt{\beta})},$$

$$1-p-q = \frac{\sqrt{\beta}}{1+3\sqrt{\beta}},$$

and when—

$$x = \frac{3+\sqrt{\beta}}{1+3\sqrt{\beta}}.$$

Thus, for a self-contained assay (one sample diluted with label, degraded, and compared with a sample of degraded label) it is possible to decide from a knowledge of the usual degraded label and background counts the proportion of the total counting time to be devoted to each of the various counts in the assay. The total counting time, T, may be estimated for any desired precision if the proportional counting times are adhered to, and the ratio x has been correctly estimated.

Under these conditions—

$$T = \frac{1}{t}\left(\frac{400}{s(1-\sqrt{\beta})}\right)^2,$$

where

$$s = \frac{(\sigma)x}{x}.$$

As it is unlikely that the ratio x has been exactly estimated before the result of the assay becomes available, a correct estimate of precision can only be made from the full formula for $\sigma(x)/x$ quoted above. Multiplying this value by the calculated result will give the standard error in the same terms as the result, *e.g.*, units per ml of benzylpenicillin for a broth, or percentage of benzylpenicillin w/w for a sample of solid penicillin.

Preparation of ^{14}C-benzylpenicillin label

The preparation of ^{14}C-labelled benzylpenicillin has been described by Sebek.[7] Our procedure, although developed separately, is similar in many respects. Phenylacetic acid (carboxyl ^{14}C) was prepared from radioactive barium carbonate* by the technique of Dauben, Reid and Yankwich[12] and converted to phenylacetamide or one of the phenacetyl derivatives used as benzylpenicillin precursors.[13] The precursor was fermented in a corn-steep liquor - lactose medium, and the harvested material filtered, extracted with butyl acetate after acidification to pH 2·0 to 2·5, and then taken up in phosphate buffer at pH 7·0 in a manner similar to that described by Sebek.[7] The phosphate buffer extract was re-extracted after acidification with amyl acetate and the N-ethylpiperidinium salt formed as described by Levy *et al.*[4] The dry N-ethylpiperidinium salt was successively reprecipitated to remove traces of non-benzylpenicillins and was then converted to potassium benzylpenicillin.[14]

The radioactivity of the prepared potassium benzylpenicillin was adjusted by the addition of non-radioactive pure potassium benzylpenicillin, so that on plating 30 mg of the pure phenylacetic acid derived from the label, an activity of 1000 to 1500 counts per minute was obtained. The dilution of the active label with non-radioactive penicillin is conveniently carried out by dissolving both materials in a suitable volume of distilled water and freeze-drying.

The penicillin label should not contain penicillins other than benzylpenicillin; this can be checked by paper-chromatographic examination.[1,2] The biological potency of the label determined by microbiological large-plate precision assay in terms of an accepted reference standard is then a measure of the benzylpenicillin content of the label. It is desirable also that the only radioactive substance present is benzylpenicillin; this can best be checked on the radioactive sample before dilution with inactive material, by the method of Lester Smith.[10]

* Radioactive phenylacetic acid can now be obtained from the Radiochemical Centre, Amersham, Bucks.

METHOD

REAGENTS—

Phosphate buffer—Dissolve 13·6 g of KH_2PO_4 and 69·6 g of K_2HPO_4 in 1 litre of water. Adjust to pH 7·0 with potassium hydroxide or phosphoric acid as necessary.

iso*Propyl ether*—Shake 2 litres of *iso*propyl ether with 30 ml of acid ferrous sulphate reagent (60 ml of recrystallised ferrous sulphate, 6 ml of concentrated sulphuric acid and 110 ml of water). Wash the ether thoroughly to remove ferrous sulphate, and then allow it to stand over sodium hydroxide pellets for 24 hours. Decant and distil, discarding the first 200 and last 400 ml of the 2-litre sample. Keep the *iso*propyl ether in a refrigerator in a brown bottle. It must give a negative test for peroxides before use.

cyclo*Hexane*—Good quality reagent grade.*

Light petroleum, boiling range 40° to 60° C—Redistil before use to remove all traces of moisture. Collect the 40° to 60° C fraction.

PROCEDURE FOR BROTH SAMPLES—

Treatment of broth samples—Filter the broth sample through a sterimat, and measure out 500 ml of filtered broth sample. Add the appropriate quantity of label calculated from the following formula—

$$\text{Weight of label} = \frac{1 + 3\sqrt{\beta}}{3 + \sqrt{\beta}} \times \frac{500}{K} \times \text{expected result},$$

where K is the potency of the label in i.u. per mg and

β is the ratio $\dfrac{\text{expected background count}}{\text{expected degraded label count}}$,

and where the expected result is expressed in units of sodium benzylpenicillin per ml.

Transfer the filtered broth to a 2-litre separating funnel, add 900 ml of chloroform and the appropriate quantity of 20 per cent. v/v phosphoric acid. (The phosphoric acid requirement is determined on a separate aliquot of filtered broth and is the amount needed to bring the pH to 2·0 to 2·2.)

Shake the mixture well for 1 minute and allow the layers to separate partly. Run the chloroform layer through a Whatman No. 54 filter-paper into a 1-litre cylinder. Collect about 700 ml of chloroform filtrate. If this volume is not available, add 50 to 100 g of anhydrous sodium sulphate[3] to the separating funnel, shake until the emulsion is broken, and collect a further quantity of chloroform filtrate.

Transfer the chloroform filtrate to a 1-litre separating funnel and extract with 50 ml of pH 7·0 phosphate buffer, then with 35 ml of buffer and finally with a further 35 ml of phosphate buffer. Pool the phosphate buffer extracts, and transfer to a 250-ml separating funnel.

Preparation of iso*propyletherate derivative*—Add 10 ml of amyl acetate to the pooled phosphate buffer extracts and sufficient 50 per cent. v/v phosphoric acid to bring the pH to 2·0 to 2·2 (determine this quantity previously on a separate aliquot of phosphate buffer). Shake immediately for 1 minute and allow the layers to separate. Run off the lower aqueous layer and collect the amyl acetate extract in a 6-inch × 1-inch test tube. Transfer the test tube to a previously prepared bath of solid carbon dioxide and acetone. Re-extract the aqueous phase with a further 10 ml of amyl acetate, and transfer the second amyl acetate extract to the test tube in the cooling bath. Prepare a glass sinter funnel of suitable size (1 to 2 inch diameter) by burying it in powdered solid carbon dioxide in a beaker. When the ice has separated in the amyl acetate extracts, rapidly filter it off by suction through the prepared sinter funnel. Remove the funnel immediately the filtration is complete to prevent water entering the amyl acetate filtrate. Transfer the amyl acetate filtrate to a 250-ml container and add 100 ml of *iso*propyl ether. Filter through a small quantity of anhydrous sodium sulphate in a filter-paper. Place the filtrate in a refrigerator for 24 to 48 hours. Decant the supernatant liquid carefully, and dry the crystals in a gentle stream of dry air.

* That made by Howards of Ilford is satisfactory.

Preparation of phenylacetic acid—Dissolve the *iso*propyletherate crystals in 6 ml of 20 per cent. w/v sodium hydroxide. Transfer the alkaline solution as completely as possible to a Pyrex-glass ampoule, seal the ampoule and place it in boiling water for 2 hours. Cool, transfer the ampoule contents to a 100-ml stoppered cylinder, and wash out the ampoule with a small quantity (say 2 × 1 ml) of water. Cautiously acidify with 7 ml of concentrated hydrochloric acid. When the solution has cooled, shake it with 40 ml of reagent-grade *cyclo*hexane, allow the layers to separate, then with a pipette take off the upper *cyclo*hexane layer. Repeat the extraction with a further three portions of 40 ml of *cyclo*hexane. Filter the pooled *cyclo*hexane extracts, through a small quantity of anhydrous sodium sulphate in a No. 54 filter-paper, into a 250-ml round-bottomed flask. Connect to a vacuum-distillation apparatus and distil off the *cyclo*hexane until about 5 ml are left. The temperature of the distilling solvent should not exceed 30° C. Blow off the remainder of the *cyclo*hexane in a gentle stream of dry air, leaving the phenylacetic acid residue in the bottom of the flask.

Dissolve the residue in 4 or 5 ml of redistilled light petroleum, warming to effect solution. Filter the warm solution through a water-jacketed glass sinter into a 10-ml round-bottomed flask, maintaining the water in the jacket at 40° to 45° C. Wash the sinter with about 2 ml of warm redistilled light petroleum. Evaporate the filtrate to dryness with a gentle stream of air blown through an efficient calcium chloride guard tube.

Dissolve the residue in about 1 ml of light petroleum with gentle heat. Cool the flask carefully in a bath of solid carbon dioxide and acetone to effect crystallisation. (NOTE—The light petroleum itself will solidify if the temperature is reduced too much.) Pour off the light petroleum from the crystals as completely as possible, redissolve them in about 1 ml of light petroleum and recrystallise. Pour off the light petroleum and place the flask in a vacuum desiccator containing paraffin wax.

Degradation of label—Weigh out about 400 mg of label and dissolve in 6 ml of 20 per cent. w/v sodium hydroxide. Process to phenylacetic acid crystals as detailed in the previous paragraph (*Preparation of phenylacetic acid*).

Radioactivity measurement—Remove the crystals from the flask and grind carefully in a small mortar. Weigh a 1-sq. cm planchette and transfer ground phenylacetic acid until exactly 30 mg are on the planchette. Press down the phenylacetic acid very lightly with the mandrel and give a slight turn before removing it. With practice it is possible to effect smooth, level plating by this technique. Reweigh the planchette to ensure that no phenylacetic acid has been lost in the process.

The times of counting for the degraded mixture, degraded label and background are derived from the following relationship—

$$\text{Total count time for degraded label, per cent.} = \frac{100}{3 + \sqrt{\beta}} \quad \ldots \quad (1)$$

$$\text{Total count time for background, per cent.} = \frac{100\sqrt{\beta}}{1 + 3\sqrt{\beta}} \quad \ldots \quad (2)$$

$$\text{Total count time for degraded mixture, per cent.} = 100 - (1 + 2),$$

where β is the ratio $\dfrac{\text{expected background count}}{\text{expected degraded label count}}$.

The total count time is dependent upon the precision required, and may be computed approximately from the formula—

$$T = \frac{1}{t} \times \left(\frac{400}{s(1 - \beta)}\right)^2,$$

where T is the total count time in minutes for all planchettes and background,
t is the degraded label count in minutes, and
s is the required standard error expressed as a percentage.

It is preferable to count at least two separately plated planchettes for both the degraded mixture and the degraded label. The individual counts should be tested for consistency by the χ^2 test, but the time devoted to either the degraded mixture or the degraded label count is the sum of the times for which the individual planchettes were counted.

PROCEDURE FOR SOLID SAMPLES—

Weigh out about 300 mg of the sample, suitably dried if necessary, and add the quantity of potassium penicillin label calculated from the relationship—

$$\text{Weight of label} = \frac{1 + 3\sqrt{\beta}}{3 + \sqrt{\beta}} \times \frac{\text{weight of sample}}{K} \times \text{expected result},$$

where K is the potency of the label in i.u. per mg.

Dissolve in 6 ml of 20 per cent. w/v sodium hydroxide solution, and proceed with the hydrolysis and extraction as described above.

CALCULATION OF THE RESULT—

For broth samples the result is calculated from the following formula—

$$\text{Units of benzylpenicillin per ml} = \left(\frac{W_L \times C_L}{C_M} - W_L\right) \times \frac{K}{500},$$

where W_L = weight of label (potassium penicillin),
C_L = degraded label count − background count,
C_M = degraded mixture count − background count, and
K = potency of label in i.u. per mg.

For solids the relationship is—

$$\text{Units of benzylpenicillin per mg} = \left(\frac{W_L \times C_L}{C_M} - Wt\right) \times \frac{K}{W}$$

where, additionally, W is the weight of sample taken, in mg.

If required, the precision of the estimate can be calculated as described previously.

RESULTS

The phenylacetic acid samples obtained from the nine experimental broths (Tables I, II and III) were subjected to radioactivity measurements by the method described. The benzylpenicillin content of each broth was then calculated from the values shown in Table IV.

TABLE IV

BENZYLPENICILLIN CONTENT OF BROTHS DESCRIBED IN TABLES I, II AND III

	Broth A			Broth B			Broth C		
	1	2	3	1	2	3	1	2	3
Radioactivity of isolated phenylacetic acid, counts per minute ..	647·3	640·5	658·1	691·6	693·6	694·5	704·1	701·2	703·7
Weight of added potassium benzylpenicillin label, mg	298·5	299·5	300·6	302·6	299·2	299·6	297·8	302·1	298·9
Volume of broth taken for assay, ml	500	500	500	250	250	250	150	150	150
Units of benzylpenicillin per ml found	1145	1170	1120	2050	2020	2015	3245	3320	3260
Units of benzylpenicillin per ml present in broth	1145	1145	1145	2050	2050	2050	3245	3285	3285
"Recovery," per cent.	100·0	102·2	97·8	100·0	98·5	98·3	98·8	101·0	99·2

Degraded label count = 1426 counts per minute.

When these results were obtained, we were using a weight of label equal to the expected weight of penicillin in the sample taken. Subsequent assays have been carried out using the ratio of label and sample calculated from the formula. A smaller standard error (about 70 per cent. of that actually obtained) would be expected if the optimum ratio of label and sample had been used.

The accuracy of the results is indicated by the percentage "recovery" figures in Table IV, which express the relationship between the amount of benzylpenicillin found by the described method and the amount actually added (see Table I). It is also possible to calculate from the results the benzylpenicillin content of the solid used to prepare the broths. This works out at 98·3 per cent. of benzylpenicillin on a weight basis.

An assay with N-ethylpiperidine[15] on the same solid gave a value of 99·1 per cent. of benzylpenicillin as a percentage of the penicillin present in the sample. In so far as the solid contained small quantities of moisture, the two values are in good agreement.

We wish to thank Dr. E. Lester Smith for suggesting the use of ^{14}C-labelled benzylpenicillin[10] in this assay method. We wish to acknowledge also the assistance of Mr. J. P. R. Tootill with the mathematical computations and the technical assistance of Miss I. Thompson.

References

1. Goodall, R. R., and Levi, A. A., *Analyst*, 1947, **72**, 277.
2. Albans, J. W., and Baker, P. B., *Ibid.*, 1950, **75**, 658.
3. Boxer, G. E., and Everett, P. M., *Anal. Chem.*, 1949, **21**, 670.
4. Levy, G. B., Fergus, G., and Caldas, J. M., *Ibid.*, 1949, **21**, 664.
5. Craig, J. T., Tyndall, J. B., and Senkus, M., *Ibid.*, 1951, **23**, 332.
6. Trenner, N. R., and Chase, R., *Perkin-Elmer Instrument News*, Summer Issue, 1951.
7. Sebek, O. K., *Proc. Soc. Exp. Biol. Med.*, 1953, **84**, 170.
8. Trenner, N. R., and Buhs, R. P., *J. Amer. Chem. Soc.*, 1948, **70**, 2897.
9. Sheehan, J. C., Mader, W. J., and Cram, D. J., *Ibid.*, 1946, **68**, 2407.
10. Smith, E. L., and Allison, D., *Analyst*, 1952, **77**, 29.
11. Kamen, M. D., "Radioactive Tracers in Biology," Academic Press, Inc., New York, 1948, p. 82.
12. Dauben, W. G., Reid, J. C., and Yankwich, P. E., "Isotopic Carbon," John Wiley & Sons, Inc., New York, 1949, p. 180.
13. Behrens, O. K., "Chemistry of Penicillin," Princeton University Press, New Jersey, 1948, p. 657.
14. Brit. Pat. 622,988, example 25.
15. Report of Analysts' Sub-Committee of Ministry of Health Conference on Differential Assay of Penicillins, *Analyst*, 1949, **74**, 79 and 550.

Glaxo Laboratories Limited
Sefton Park
Stoke Poges, Bucks.

July 28th, 1954

Reprinted from Chemistry and Industry, 1955, *p.* 1183

GRISEOFULVIN AND DIMETHYLFORMAMIDE

By G. C. Ashton and A. Rhodes

Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks.

Griseofulvin, discovered in 1939 as a metabolic product of *Penicillium griseofulvum* Dierckx by Oxford, Raistrick and Simonart,[1] was subsequently shown by Brian, Wright, Stubbs and Way[2] to be of potential use as a systemic "fungicide" against certain plant diseases. Evaluation of griseofulvin as an agricultural chemical has hitherto been handicapped by limitation of supply, due to poor fermentation yields, and by difficulties in formulation, analysis and methods of control resulting from the low solubility of griseofulvin in most organic solvents, particularly those miscible with water.

The first difficulty has recently been overcome as a result of the production of griseofulvin in substantial amounts by submerged fermentation, and the second problem has been resolved by the finding that as a solvent for griseofulvin, *NN'*-dimethylformamide has novel properties, which are outlined in this communication.

We find that griseofulvin is soluble in *NN'*-dimethylformamide to the extent of 12–14% (w/v) at room temperature and can be precipitated from concentrated solutions with water to yield fine dispersions. Stable liquid concentrates of griseofulvin, suitable for agricultural, medical or veterinary use, can thus be conveniently prepared by mixing *NN'*-dimethylformamide solutions containing, for example, 8–10% griseofulvin with appropriate quantities of a suitable wetting-out agent.

Solutions of griseofulvin in *NN'*-dimethylformamide up to about 1% (w/v) can be infinitely diluted with water without precipitation of griseofulvin. Such solutions can be utilized in the u.-v. spectrophotometric assay[3] of griseofulvin: the extinction value of pure griseofulvin in 0·2% aqueous *NN'*-dimethylformamide at 295·7 mμ is 705.

Addition of 8 volumes of water to *NN'*-dimethylformamide solutions of griseofulvin containing more than 2·5% (w/v) results in almost quantitative precipitation of griseofulvin. In this way we have been able to effect considerable purification of crude griseofulvin, 60–70% material being converted to a purity of 90–95% by one precipitation. This phenomenon has been utilized in an isotope-dilution assay[3] for griseofulvin.

Finally, *NN'*-dimethylformamide is a suitable solvent for the polarimetric assay of griseofulvin: the specific rotation of pure griseofulvin in *NN'*-dimethylformamide at 25° C. is + 364°, and the rotation remains unchanged for at least 24 hours.

NN'-dimethylacetamide behaves towards griseofulvin like *NN'*-dimethylformamide, but formamide and diethylformamide have a much lower solubility for the antibiotic.

A number of the findings outlined above are the subjects of patent applications.

Received August 16, 1955

References

[1] Oxford, A. E., Raistrick, H. & Simonart, P., *Biochem. J.*, 1939, **33**, 240

[2] Brian, P. W., Wright, J. M., Stubbs, J. & Way, A. M., *Nature, Lond.*, 1951, **167**, 347

[3] Ashton, G. C., Brown, A. P. & Tootill, J. P. R. (in preparation)

[9551] PRINTED BY HARRISON AND SONS, LTD., ST. MARTIN'S LANE, LONDON

(38)

Determination of Grisofulvin in Fermentation Samples

Part I. Spectrophotometric Assay

BY

G. C. ASHTON and A. P. BROWN

Appendix to Part I: Seven-point Correction Procedure

BY

G. C. ASHTON and J. P. R. TOOTILL

Reprinted from THE ANALYST, *the Journal of the Society for Analytical Chemistry, April*, 1956, *Vol.* 81, *No.* 961, *pp.* 220–227

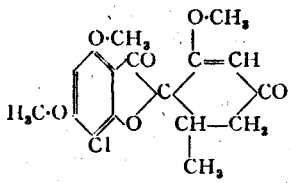
Determination of Griseofulvin in Fermentation Samples

Part I. Spectrophotometric Assay

By G. C. ASHTON and A. P. BROWN

A physico-chemical procedure for the determination of griseofulvin in fermentation broth samples is described. The method involves solvent extraction of the whole-broth sample with butyl acetate and measurement of the ultra-violet spectrophotometric absorption of the extract. A correction procedure is described (in the Appendix to Part I) that allows mathematically for irrelevant ultra-violet absorption in the extract.

Griseofulvin was first isolated by Oxford, Raistrick and Simonart[1] from *Penicillium griseofulvum* Dierckx grown in surface culture. The structural formula of griseofulvin determined by Grove, MacMillan, Mulholland and Rogers[2] is shown below—



Brian, Curtis and Hemming[3,4] later isolated the same substance from several different *Penicillium* spp. More recently it has been found possible to grow griseofulvin-producing penicillia under deep fermentation conditions, with considerably increased yields.

The development of such fermentation procedures, together with the increasing interest in the agricultural application of griseofulvin as a systemic fungicide, has made it necessary to have suitable assay procedures for control and standardisation. Brian *et al.*[3] have described a biological assay based on the curling of *Botrytis allii* hyphae in the presence of griseofulvin. However, this assay is rather imprecise in our experience and difficult to apply routinely. This paper describes the development of a spectrophotometric assay, suitable for control purposes, based on the absorption spectrum of griseofulvin.

Experimental

Extinction values of griseofulvin—

The ultra-violet absorption spectrum of griseofulvin in various solvents is shown in Fig. 1. Grove *et al.*[5] quote a logϵ of 4·34 at 291 mμ in methanol, corresponding to an $E^{1\%}_{1cm}$ at that wavelength of 621 (molecular weight of griseofulvin is 352·5). We had obtained values higher than this, and a quantity of very pure griseofulvin was therefore prepared to establish the extinction values in various solvents.

Griseofulvin with an extinction value of 670 on our instrument at 295·7 mμ in 0·2 per cent. aqueous dimethylformamide solution was dissolved in benzene at room temperature until a saturated solution was obtained. The benzene was progressively evaporated at room temperature in a stream of dry air. Colourless crystals of griseofulvin were deposited on the walls of the container: they were washed with benzene, the solvent was evaporated under reduced pressure, and the crystals were dried at 100° C for 2 hours. The extinction value of this material at 295·7 mμ in 0·2 per cent. aqueous dimethylformamide was 688 on our instrument.

A quantity of this material was sublimed at 210° C at atmospheric pressure. The extinction value of the sublimate at 295·7 mμ was also 688 on our instrument. Several preparations from the original starting material gave the same values.

The instrument for determining these extinction values was a much-used Unicam SP500. Although assays on this instrument could be compared with the extinction values established

on the same instrument, for inter-laboratory comparison it was desirable to obtain an absolute value for the extinction of pure griseofulvin in one solvent. The instrument was therefore standardised with 0·005 per cent. of potassium dichromate in 0·1 *N* sulphuric acid as described by Cama, Collins and Morton.[6]

The corrected value for the extinction value of griseofulvin in 0·2 per cent. aqueous dimethylformamide is 705 at 295·7 mμ. This value was confirmed independently on a sample of sublimed griseofulvin on two Hilger Uvispek absorptiometers in good condition, values of 702 and 705 being obtained at 295·5 to 296·0 mμ on freshly prepared solutions.

The extinction value of pure griseofulvin in butyl acetate was found to be 705 at 289·25 mμ. The extinction value in methanol is approximately 680 at 291·5 mμ, but in our experience griseofulvin is not stable in this solvent.

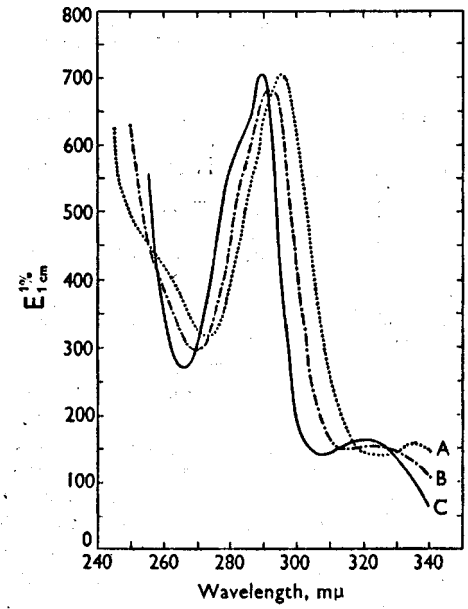


Fig. 1. Ultra-violet absorption of griseofulvin in different solvents. Curve A, in 0·2 per cent. aqueous dimethylformamide; curve B, in methanol; curve C, in butyl acetate

SOLVENT EXTRACTION OF FERMENTATION MATERIAL—

At least 80 per cent. of the griseofulvin in fermentation broth samples is in the mycelium, from which it is necessary to extract it; for doing this several solvents were tried in preliminary experiments. Aliquots of whole broth were extracted with four volumes of the solvent three times, the extracts then being pooled and diluted to 100 ml. One-ml quantities of the extracts were evaporated to dryness in a stream of dry air and the residues dissolved in 5 ml of methanol. The amount of griseofulvin in the methanol extracts was determined. The results are shown in Table I.

TABLE I

GRISEOFULVIN EXTRACTED BY DIFFERENT SOLVENTS

Solvent	Griseofulvin in methanol extract, μg per ml
Butyl acetate	6·3
Methyl Cellosolve	7·3
Dimethylformamide	6·8
Methanol	7·1
*cyclo*Hexane	0·8
Ether	6·2
Chloroform	6·4
Benzene	5·1

The results in Table I are not corrected for irrelevant absorption (see below) and except for the *cyclo*hexane extract the results are not essentially different. Of the solvents examined, butyl acetate proved to be most convenient in practice, being relatively immiscible with whole broth and transmitting in ultra-violet light down to 270 mμ. Most of the other solvents gave emulsions on shaking with whole broth.

Various extraction procedures with butyl acetate and whole broth were tried, including mixing in a Waring Blendor, manual shaking in a separating funnel and manual and mechanical shaking in a boiling tube. Repetitive extraction of a small volume (2 to 10 ml) of whole broth in a boiling tube with four volumes of butyl acetate proved satisfactory. Altering the pH of the broth had no effect on the amount of griseofulvin extracted.

The number of extractions required to effect complete removal of griseofulvin was determined. Two broth samples were extracted by shaking one volume of each sample with four volumes of butyl acetate three times. The extracts were assayed spectrophotometrically, the seven-point correction procedure being used (see below). The results are shown in Table II.

TABLE II

NUMBER OF BUTYL ACETATE EXTRACTIONS REQUIRED

Sample	Extraction	Griseofulvin content of extract, μg per ml	Percentage of total extracted
26Q1	First	214·50	94·6
26Q1	Second	10·95	4·8
26Q1	Third	1·26	0·6
26Q2	First	184·86	95·2
26Q2	Second	8·65	4·4
26Q2	Third	0·71	0·4

It is apparent that two extractions remove more than 99 per cent. of the available griseofulvin. The third extraction removed more impurities than griseofulvin, as evidenced by the large correction value required on the third extract; the non-corrected and corrected absorbances at the peak in each instance were 0·560 and 0·041 (26Q1), 0·347 and 0·049 (26Q2).

CORRECTION PROCEDURE—

A true estimate of the griseofulvin content of the butyl acetate extract can only be obtained if the absorption curve is corrected for components other than griseofulvin, that is, for irrelevant absorption. If this absorption were of equal magnitude over a suitable range,

TABLE III

REPRODUCIBILITY OF SPECTROPHOTOMETRIC ASSAY AS APPLIED TO FERMENTATION SAMPLES

Sample	Diluted extract assay		
	a, μg per ml	*b*, μg per ml	*c*, μg per ml
R28/R1	9·17	9·20	9·03
R27/R2	7·73	7·42	7·46
PC1	8·27	7·96	8·34
PC2	8·60	8·56	8·43
IDI	8·84	8·98	8·70
Q36/Q1	5·17	5·25	5·33
Q36/Q2	8·00	7·92	8·00
Q38/Q1	8·79	8·71	9·50
Q38/Q4	6·13	6·25	6·17
Q38/Q2	6·25	6·18	6·22
R39/R2	6·65	7·02	7·16

say 289 to 325 mμ, the griseofulvin content could be calculated by subtracting the 322·5-mμ peak value from the 289-mμ peak value and relating this difference to the corresponding difference for pure griseofulvin. In practice, a useful estimate of the approximate potency of a broth sample can be obtained in this way when previous experience has shown that the medium and organism employed produce ultra-violet absorbing components that are mainly griseofulvin.

A correction procedure described by Morton and Stubbs[7] and widely applied in the spectrophotometric determination of vitamin A was examined in relation to the griseofulvin determination. For reasons discussed in the Appendix to Part I, this three-point correction procedure was not considered suitable, and an alternative procedure based on readings at seven wave-lengths was developed.

PRECISION OF SPECTROPHOTOMETRIC ASSAY—

Several broth samples were assayed in triplicate by the method described, with correction for irrelevant absorption by the seven-point procedure. The results are shown in Table III.

The standard deviation of a single determination on these fermentation samples averaged ± 2·4 per cent.

The purity of a number of broth solids was determined spectrophotometrically under routine conditions. Griseofulvin is not very soluble in the common organic solvents, whereas it is readily soluble and stable in dimethylformamide.[8]

Approximately 50 mg of the griseofulvin solid were dissolved in 25 ml of dimethylformamide, in duplicate. From each solution two 5-ml portions were each diluted to 1 litre with butyl acetate. The griseofulvin contents of all four dilutions were then determined spectrophotometrically by the seven-point correction procedure. Some results are shown in Table IV.

TABLE IV

PRECISION OF SPECTROPHOTOMETRIC ASSAY AS APPLIED TO SOLID PREPARATIONS

Sample	Purity after first weighing and primary dilution		Purity after second weighing and primary dilution	
	First dilution, %	Second dilution, %	First dilution, %	Second dilution, %
DB84	98·0	98·5	96·0	96·5
DB117	95·9	95·8	97·4	96·0
DB118	99·8	101·2	98·7	99·9
DB126	86·2	86·1	87·2	87·1
DC300	87·2	88·4	88·4	89·3
DC450	86·0	84·3	85·7	86·5
DD83	98·2	98·0	96·6	96·4
DD134	95·7	96·0	94·4	94·0

A single determination based on one weighing and one dilution has a standard deviation averaging ± 1·6 per cent., this error being composed partly of errors due to weighings and primary and secondary dilutions and partly of the error of the spectrophotometric measurements.

METHOD

PROCEDURE—

Measure 5 ml of well mixed whole-broth sample into a 6-inch × 1-inch boiling tube. Add 20 ml of reagent grade butyl acetate, stopper the tube and shake hard for 2 minutes. Decant as much of the butyl acetate extract as possible; re-extract with a further 20-ml quantity of butyl acetate. Combine the two extracts and dilute to 100 ml with butyl acetate. Filter a quantity of the diluted extract through a No. 54 Whatman filter-paper.

Dilute an aliquot of the filtered extract with butyl acetate to give a solution containing between 5 and 15 μg of griseofulvin per ml.

Measure the ultra-violet absorption of this dilute solution against butyl acetate at 288, 290, 292, 294, 296, 298 and 300 mμ in 1-cm silica cells. It is not necessary to know the blank values of the cells, but they should be uniform or linear over the wavelength range employed.

CALCULATION—

The calculation of the result from the spectrophotometric measurements is based on quadratic curvature corrected coefficients. The calculation of these coefficients is described in the Appendix to Part I.

The absorbance at each of the seven wavelengths is multiplied by the coefficient for that wavelength, the products are summed with due regard to algebraic sign, and the sum of the products is divided by the divisor. The result is in terms of μg of griseofulvin per ml

in the solution measured on the spectrophotometer. The result is then multiplied by 20 to allow for the broth volume (5 ml) and the extract volume (100 ml) and by any dilution factors involved in preparing the solution for spectrophotometry. An example to illustrate the calculation is as follows—

Column I wavelength	Column II absorbance	Column III coefficient	Column IV coefficient × absorbance
288	0·562	− 10·738	− 6·034756
290	0·568	+ 10·872	+ 6·175296
292	0·494	+ 11·478	+ 5·670132
294	0·372	− 1·612	− 0·599664
296	0·253	− 11·682	− 2·955546
298	0·176	− 7·644	− 1·345344
300	0·134	+ 9·326	+ 1·249684
		Total	2·159802

Divisor = 0·2585.

Therefore, concentration of measured solution $= \frac{2{\cdot}159802}{0{\cdot}2585} = 8{\cdot}36\ \mu g$ per ml.

Broth titre $= 8{\cdot}36 \times \frac{100}{5} \times$ butyl acetate dilution factor.

Results

Some results obtained by the spectrophotometric assay are discussed in Part II.

The authors are indebted to Miss I. Thompson and Mrs. E. Hockley for technical assistance, and to Mr. W. H. C. Shaw for measurement of some extinction values on samples of pure griseofulvin.

References

1. Oxford, A. E., Raistrick, H., and Simonart, P., *Biochem. J.*, 1939, **33**, 240.
2. Grove, J. F., MacMillan, J., Mulholland, T. P. C., and Rogers, M. A. T., *J. Chem. Soc.*, 1952, 3977.
3. Brian, P. W., Curtis, P. J., and Hemming, H. G., *Trans. Brit. Mycol. Soc.*, 1946, **29**, 173.
4. ——, ——, ——, *Ibid.*, 1949, **32**, 30.
5. Grove, J. F., MacMillan, J., Mulholland, T. P. C., and Rogers, M. A. T., *J. Chem. Soc.*, 1952, 3949.
6. Cama, H. R., Collins, F. D., and Morton, R. A., *Biochem. J.*, 1952, **50**, 50.
7. Morton, R. A., and Stubbs, A. L., *Analyst*, 1946, **71**, 348.
8. Ashton, G. C., and Rhodes, A., *Chem. & Ind.*, 1955, 1183.

Glaxo Laboratories Ltd.
Sefton Park
Stoke Poges, Bucks.

July 27th, 1955

Determination of Griseofulvin in Fermentation Samples

Appendix to Part I: Seven-point Correction Procedure

By G. C. ASHTON and J. P. R. TOOTILL

In the spectrophotometric determination of griseofulvin in fermentation samples, it is necessary to correct the ultra-violet absorption values of butyl acetate extracts for irrelevant absorption, that is, for absorption not due to griseofulvin itself. This problem is a general one with ultra-violet spectra of impure preparations or extracts, and a commonly applied correction procedure is that due to Morton and Stubbs[1] for vitamin A in fish oils. Their three-point correction assumes that three wavelengths can be found at which the irrelevant absorption can be joined by a straight line. Such an approach is empirical and the choice of suitable wavelengths difficult.[2]

In our opinion a better method would be to apply a correction procedure that is determined by the shape of the irrelevant-absorption curve and corrects for it. This procedure can only be used when the form of the irrelevant absorption is less complex than that of the substance being assayed; fortunately, with griseofulvin extracts we have found that the irrelevant absorption with material from the medium and strain employed gives a quadratic curve.

Determination of the shape of the irrelevant-absorption curve—

Preliminary experiments were carried out to establish the reference curve obtained with pure griseofulvin in butyl acetate at 2-mμ intervals with a high degree of precision. On any one replicated experiment a very small error was obtained, but it was not found possible to reproduce the reference absorption curve subsequently within this error. It seemed probable that this difficulty was due to slight differences in wavelength setting from day to day, coupled with the very steep slope of both approaches to the griseofulvin peak at 289·25 mμ. Consequently, in the mathematical analysis of broth-extract absorbances in butyl acetate, it was not possible to determine the shape of the irrelevant-absorption curve by comparison with a previously established reference curve.

When standard solution and butyl acetate extract were read side by side, each at the same wavelength scale settings, this difficulty was overcome and it was possible to analyse the results mathematically. Typical results are shown in Table I.

Table I

Analysis of variance of results shown graphically (Fig. 2)

Source of variation	Degrees of freedom	Mean square
Uncorrected griseofulvin estimate	1	2·764,139,665
Correction for uniform absorption	1	0·007,478,159
Additional correction for linear absorption ..	1	0·000,090,070
Additional correction for quadratic absorption ..	1	0·000,207,345
Additional correction for cubic absorption ..	1	0·000,001,089
Residual absorption	4	0·000,000,918

The standard error per observation is known to be approximately 0·001 absorbance unit; hence neither the cubic correction nor the residual error indicate any significant absorption of higher complexity than quadratic.

The nature of the irrelevant absorption in this particular sample is shown in Fig. 2. Although in such precise work as that in the experiment summarised above the wavelength setting was a critical factor, it was found experimentally that good reproducibility could be obtained in practice by establishing a set of coefficients describing the standard curve and using these to correct for irrelevant absorption in broth extracts (Table III, see below).

BASIS OF QUADRATIC CORRECTION COEFFICIENTS AND CHOICE OF WAVELENGTHS—

Irrelevant absorption can be corrected for by developing a set of coefficients related to the absolute values of the reference ultra-violet absorption curve but independent of uniform, linear or quadratic absorption. The derivation of such coefficients is shown below by reference to a set of experimental results.

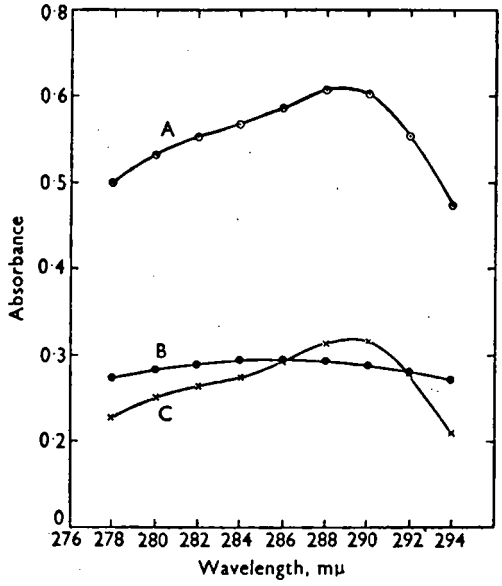


Fig. 2. Irrelevant absorption in butyl acetate extracts containing griseofulvin. Curve A, total absorption of butyl acetate extract; curve B, irrelevant absorption in extract; curve C, curve due to griseofulvin in extract

The number of wavelength readings, the spacing of the readings and the position of the chosen wavelengths in relation to the reference curve require consideration. Although quadratic irrelevant absorption can be detected and allowed for with four points, a rather imprecise result would be obtained unless the readings were replicated. A larger number of wavelength readings is therefore desirable and, although any number could be used, seven readings were chosen. A wavelength spacing of 2 mμ seemed a suitable value with our instrument (Unicam SP500 quartz spectrophotometer).

The choice of wavelengths was decided by establishing values for a standard solution at 278 to 302 mμ in steps of 2 mμ. Successive groups of seven wavelength readings were compared with the aid of orthogonal polynomials and that portion of the reference curve with the highest cubic component was chosen. The seven wavelengths thus found were 288 to 300 mμ in steps of 2 mμ.

CALCULATION OF QUADRATIC CORRECTION COEFFICIENTS—

The mechanics of calculating the coefficients is best described by reference to an example (Table II).

The reference solutions are prepared in triplicate by dissolving approximately 10 mg of pure griseofulvin in 5 ml of dimethylformamide and diluting to 1 litre with butyl acetate.

REPRODUCIBILITY OF RESULTS WITH CORRECTION COEFFICIENTS

As pointed out above, the reference absorption curve is not reproducible from day to day within the small limits of error of a single reading on the instrument. However, in practice it is not convenient to read a standard and unknown side by side at seven wavelengths and calculate an individual set of coefficients for such readings. Thus a compromise is necessary, resulting in some loss in accuracy but a great saving in computational labour. The effect of computing the griseofulvin concentration of four butyl acetate extracts measured individually against four separately determined sets of correction coefficients is shown in Table III.

TABLE II

CALCULATION OF COEFFICIENTS CORRECTING FOR QUADRATIC CURVATURE

Wavelength, mμ	Standard solutions absorbances = S: 9·811 μg per ml	10·031 μg per ml	10·080 μg per ml	I Sum of absorbances of standard solutions ΣS	II Linear coefficients X_1	III I × II $X_1\Sigma S$	IV Curvature* coefficients X_2	V I × IV $X_2\Sigma S$	VI Correction coefficients (formula below)
288	0·656	0·668	0·672	1·996	− 3	− 5·988	+ 5	+ 9·980	− 10·738
290	0·663	0·673	0·679	2·015	− 2	− 4·030	0	—	+ 10·872
292	0·580	0·590	0·592	1·762	− 1	− 1·762	− 3	− 5·286	+ 11·478
294	0·437	0·442	0·445	1·324	0	—	− 4	− 5·296	− 1·612
296	0·298	0·300	0·302	0·900	+ 1	+ 0·900	− 3	− 2·700	− 11·682
298	0·206	0·207	0·209	0·622	+ 2	+ 1·244	0	—	− 7·644
300	0·156	0·159	0·161	0·476	+ 3	+ 1·428	+ 5	+ 2·380	+ 9·326
Sums of columns I to VI				(ΣS) = 9·095 (ΣS^2) = 14·325321	Zero	($X_1\Sigma S$) = − 8·208	Zero	($X_2\Sigma S$) = − 0·922	Zero

NOTES—

1. The values in column VI are computed at each wavelength from the formula—
$$84\Sigma S - 12(\Sigma S) - 3X_1(X_1\Sigma S) - X_2(X_2\Sigma S).$$
2. The divisor is computed from the formula: $\dfrac{84(\Sigma S^2) - 12(\Sigma S)^2 - 3(X_1\Sigma S)^2 - (X_2\Sigma S)^2}{\text{sum of solution concentrations in } \mu g \text{ per ml}} = 0{\cdot}2585,$
where (ΣS^2) is the sum of the squares of the values in column I.

CHECKS—

(*a*) The correction coefficients (column VI) should sum to zero.
(*b*) Multiply the values in column I by the appropriate coefficient in column VI, sum the products algebraically, and divide by the calculated divisor. The result should be the sum of the concentrations of the three standard solutions in μg per ml.

* From Fisher and Yates.[3]

TABLE III

REPRODUCIBILITY OF RESULTS WITH SEPARATELY DETERMINED CORRECTION COEFFICIENTS

Butyl acetate extract	Correction coefficients: *a*	*b*	*c*	*d*
Q1 ..	8·36	8·30	8·59	8·38
Q2	7·95	7·91	8·19	7·98
F1	8·41	8·37	8·66	8·44
F2	8·16	8·12	8·40	8·19

Figures represent griseofulvin content of butyl acetate extracts in μg per ml.

It can be seen that the error involved by this compromise procedure is of the same order as that between replicate broth extracts (Part I, Table III, p. 222).

DISCUSSION

Owing to the difficulty of reproducing the reference curve already mentioned, it has not been possible to ascertain exactly the optimal portion of the curve to use. It may be that more exhaustive examination would indicate a different choice of wavelengths.

A further discussion of the theoretical basis for this correction procedure, and of the precision of this correction, is given in the paper by Ashton and Tootill[4] in this issue.

REFERENCES

1. Morton, R. A., and Stubbs, A. L., *Analyst*, 1946, **71**, 348.
2. Gridgeman, N. T., *Ibid.*, 1951, **76**, 449.
3. Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," Fourth Edition, Oliver & Boyd, Edinburgh, 1953, p. 80 *et seq.*
4. Ashton, G. C., and Tootill, J. P. R., *Ibid.*, 1956, **81**, 232.

GLAXO LABORATORIES LTD.
SEFTON PARK
STOKE POGES, BUCKS.

July 27th, 1955

(39)

Determination of Grisofulvin in Fermentation Samples

Part II. Isotope-dilution Assay

BY

G. C. ASHTON

Reprinted from THE ANALYST, *the Journal of the Society for Analytical Chemistry, April*, 1956, *Vol.* 81, *No.* 961, *pp.* 228–231

Determination of Griseofulvin in Fermentation Samples

Part II: Isotope-dilution Assay

By G. C. ASHTON

To confirm the results of the spectrophotometric assay (Part I), and also to provide an alternative procedure for the determination of griseofulvin, an isotope-dilution assay based on chlorine-36 griseofulvin has been developed. Results obtained by this procedure are compared with those by the spectrophotometric method.

The choice of tracer for the preparation of the labelled griseofulvin lay between carbon-14 and chlorine-36. It was found that radioactive chlorine was readily incorporated into the griseofulvin molecule in good yield and so this tracer was chosen. It has the further advantage that material so labelled can be distinguished from dechlorogriseofulvin,[1,2] which may be present, by alumina chromatography.[2]

Preparation of chlorine-36 griseofulvin "label"—

The chlorine-36 griseofulvin was prepared by submerged fermentation with the strain and medium described elsewhere[3]; this strain does not produce dechlorogriseofulvin in submerged culture under a variety of conditions. The medium contained $K^{36}Cl$.*

The harvested broth volume was measured (200 ml) and the whole broth extracted four times with an equal volume of ether. The pooled ether extracts were filtered through a Whatman No. 54 filter-paper. Dimethylformamide (20 ml) was added to the filtered ether extract and the ether was distilled from a water bath or low-temperature hot-plate. The dimethylformamide residue was treated with 160 ml of water and the precipitated griseofulvin filtered off. The precipitate was dissolved in dimethylformamide and a sufficient quantity of carrier (99·9 per cent. pure griseofulvin) added to bring the activity of the final isolated material to approximately 0·1 μC per g. The volume of dimethylformamide used was sufficient to give a 5 per cent. concentration of griseofulvin; eight volumes of water were then added to reprecipitate the "diluted" griseofulvin. The precipitate was filtered off, dried and dissolved in the minimum quantity of warm benzene. The benzene solution was filtered and evaporated at room temperature to a small volume in a stream of dry air. Griseofulvin crystallised as the volume decreased and was successively removed from the mother liquors. The crystalline material was freed from benzene by heating at 100° C to constant weight. The final product was 99·8 per cent. pure by seven-point spectrophotometric assay, with a m.p. 221° to 222° C. It was free from dechlorogriseofulvin when tested by MacMillan's nitric acid test.[2] About 10 g of suitably diluted material were obtained from 10 μC of chlorine-36 as $H^{36}Cl$.

The specific activity of the non-diluted griseofulvin was found to be 5·175 and 5·023 μC per g in two series of fermentations; 45·7 and 44·3 per cent. of the respective preparations were labelled with chlorine-36. The percentage of added chlorine-36 taken up from the medium into the griseofulvin produced was 19·3 per cent. in one preparation and 32·6 per cent. in another.

Experimental

The experimental work associated with an isotope-dilution assay is essentially concerned with the development of a small-scale extraction process to yield either the diluted label in pure form or else a pure degradation product containing the isotopically labelled moiety. In this instance it was found possible to isolate griseofulvin free from contaminants and in a form suitable for counting, so that recourse to the preparation of a derivative was not necessary.

* The chlorine-36 was purchased from the Radiochemical Centre, Amersham, Bucks., in the form of 2N hydrochloric acid, 1·0 ml of which contained 8 μC of chlorine-36.

EXTRACTION PROCEDURE—

The experimental procedure envisaged was to extract a volume of whole broth containing 100 to 150 mg of griseofulvin with a suitable solvent, add 50 mg of labelled material, distil off the solvent to a low volume, precipitate the diluted "label" and purify on an alumina column.

Butyl acetate was examined, as it had proved suitable for the spectrophotometric assay. However, it was found that on vacuum-distillation griseofulvin was slightly degraded in this solvent in the presence of dimethylformamide. Of other solvents examined ether was found to be satisfactory, no destruction of griseofulvin occurring on boiling it off. The extraction efficiency from whole broth into an equal volume of ether was found to be 70 per cent., indicating that four extractions with an equal volume of ether would remove over 99 per cent. of the griseofulvin present.

It was convenient to boil off the ether in the presence of a small volume (2 to 5 ml) of dimethylformamide, as the griseofulvin remained in this solvent and could then be precipitated with at least four volumes of water, leaving only 2 to 4 per cent. of the griseofulvin in solution.

It is, of course, essential that the isolated griseofulvin sample for counting should be free from dechlorogriseofulvin. In the absence of this latter substance in the fermentation (which can be checked by isolating a small sample and testing by the nitric acid test[2]) the chromatographic procedure described by Grove *et al.*[4] is excellent for purifying fermentation extracts. If dechlorogriseofulvin occurs in the fermentation sample to be assayed, the chromatographic separation described by MacMillan[2] must be applied. In the method described below it is assumed that dechlorogriseofulvin is absent, and the alumina chromatographic procedure employed is essentially that of Grove *et al.*[4] It was necessary to use an all-glass column to prevent the isolated material from becoming discoloured.

The solvent was readily removed in a stream of dry air at room temperature. The final traces of solvent could be removed by heating the solid at 100° C. Destruction of the griseofulvin did not take place at this temperature, neither was the radioactivity of the solid affected by this treatment.

The purity of the isolated solid may be checked by the seven-point spectrophotometric assay. A sample of "label" was taken through the extraction process; its radioactivity was unchanged by the manipulations involved.

COUNTING PROCEDURE—

Chlorine-36 is a medium-energy β-particle emitter, and as such may be counted in a liquid or end-window counter. We prefer to use an end-window counter, as it gives, in our experience, better replication of counts and a lower specific activity label may be used. With a 1 sq.cm planchette and a G.E.C. type EHM2S Geiger - Müller tube, 30 mg of label of specific activity 0·1 μC per g gave a count of about 1300 to 1400 counts per minute. The weight of griseofulvin on the planchette should be $30 \pm 0{\cdot}1$ mg, as this weight is not on the "infinite thickness" part of the response curve. It was found that replicate platings gave counts agreeing within 2 per cent. or less by this procedure.

ACCURACY AND PRECISION OF THE ISOTOPE ASSAY—

The determination of a substance by dilution with a radioactive one is a fundamentally accurate procedure provided due attention is paid to the purity of the label and isolate. However, the precision of the measurement of the isotope dilution is dependent on the ratio of sample to label, on the relative times allocated to the label, sample and background counts and on the total count time. The conditions for obtaining the best precision have been discussed elsewhere.[5,6]

METHOD

PROCEDURE—

Extract a volume of whole broth containing 100 to 150 mg of griseofulvin four times with an equal volume of ether. The ether extraction must be quantitative; the isotope dilution is based on the amount of griseofulvin extracted.

Pool the ether extracts and filter through a Whatman No. 54 filter-paper. Dissolve approximately 50 mg of griseofulvin label in 5 ml of dimethylformamide in a 250-ml beaker. Put 100 ml of the filtered ether extract into the beaker and distil off the ether on a water bath, replenishing the beaker contents as necessary until all the ether has been boiled off.

Cool the dimethylformamide residue and add 8 to 10 volumes of water. Set the precipitate aside overnight, and then filter through a small Buchner funnel containing a Whatman No. 54 filter-paper. Dry the precipitate at 100° C and dissolve the dried precipitate in 3 ml of benzene by warming in a water bath.

In the absence of dechlorogriseofulvin (see p. 229) prepare an alumina column 1 cm in diameter and 10 to 12 cm high by pouring in a slurry of activated alumina in a mixture containing 99 per cent. of benzene and 1 per cent. of ethanol. The activated alumina is prepared by washing Peter Spence grade 0 alumina to pH 4·0 to 4·5 with sulphuric acid, washing with water until the washings are free of sulphate and heating the filtered acid-washed alumina at 150° C overnight. Drain the column to $\frac{1}{4}$ inch above the surface and apply the benzene solution of griseofulvin. Develop the column with a mixture containing 99 per cent. of benzene and 1 per cent. of ethanol until the griseofulvin, which fluoresces bright blue in ultra-violet light, is eluted. The griseofulvin-containing eluate should then be evaporated in a stream of dry air at room temperature.

Dissolve the benzene residue in 2 ml of dimethylformamide. Filter through a small Hirsch filter with a Whatman No. 54 filter-paper into a 50-ml centrifuge tube. Add 25 to 30 ml of water, centrifuge the contents and decant. Dry the precipitate at 150° C, cool, and wash twice with a small quantity of dry ether to remove any residual dimethylformamide.

Radioactivity determination—

The dried precipitate should be colourless; the purity, which should be determined by the spectrophotometric procedure (Part I), should not be less than 98 per cent. The material should be free from dechlorogriseofulvin by the nitric acid test.[2]

Grind the dried residue in a small mortar to a fine powder, and place 30 ± 0·1 mg on a 1 sq. cm planchette; level the surface with a suitably sized steel mandrel. Determine the radioactivity of the prepared planchette by means of a lead castle and suitable end-window counter (*e.g.*, a G.E.C. type EHM2S).

At the same time prepare a similarly weighted planchette of the griseofulvin-label, and determine the radioactivity of this planchette also.

Calculation—

The griseofulvin content of the whole broth sample is given by—

$$\left(\frac{C_L \times W_T}{C_M} - W_T\right) \times \frac{1000}{V} = \mu g \text{ of griseofulvin per ml,}$$

where C_L = the count of the label (counts per minute), less the background count,
C_M = the count of the isolated griseofulvin sample, less the background count,
W_T = the weight of label used in mg, and
V = the volume of whole broth in ml.

The precision of a single isotope assay of this type can be calculated if necessary.[6]

Results

Comparison of isotope-dilution and spectrophotometric assays—

The griseofulvin content of a number of whole-broth samples was determined in duplicate by the isotope assay and compared with results obtained spectrophotometrically, see Table I.

The values in Table I are consistent within the limits of error of both assays.

Because most of the griseofulvin of whole broth is located in the mycelium, it is not possible to carry out "recovery assays." Thus the validity of either assay method lies in the consistency of results obtained by the two methods.

Several comparisons between the spectrophotometric assay and the bio-assay of Brian *et al.*[7] were made. Thus aliquots of the butyl acetate extracts from the spectrophotometric assay found by this assay to contain 210 μg were evaporated at room temperature. The residues were dissolved in 1 ml of dimethylformamide and diluted with 20 ml of water. The resulting solutions were bio-assayed against a standard containing 10 μg of pure griseofulvin per ml. In all tests carried out the butyl acetate extracts and the standard gave curling at the same dilution within the rather wide limits of error of the method.

Discussion

For routine purposes the spectrophotometric assay with the seven-point correction procedure is suitable. The major object of developing an isotope-dilution procedure was

TABLE I

ISOTOPE-DILUTION AND SPECTROPHOTOMETRIC ASSAY RESULTS

Sample	Isotope-dilution assay: griseofulvin in sample taken		Spectrophotometric assay: griseofulvin in corresponding volume of sample, mg
	Assay 1, mg	Assay 2, mg	
36Q1/9	59·0	51·0	56·0
36Q1/11	37·1	40·0	44·8
36Q2/11	79·0	79·0	82·5
36Q1/14	34·0	35·5	39·5
36Q2/14	81·0	85·5	86·5
36Q1/16	80·3	81·7	78·9
38Q4/15	65·7	67·1	66·6
39R2/11	96·0	88·5	83·5
38Q2/18	76·0	77·5	74·5
38Q1/20	108·0	96·5	109·2
39R2/14	108·0	118·3	109·5

to verify results got by the spectrophotometric assay. Thus whenever it is necessary to confirm a spectrophotometric assay result, for example, in work involving a new medium or a new strain, the isotope assay is valuable.

Various other assay procedures have been examined, including a polarimetric assay based on the high positive dextrorotation of griseofulvin, and several colorimetric procedures. Of the colorimetric procedures the rather non-specific reaction with sulphuric acid[4] proved unsuitable, owing to the presence of interfering substances in all extracts except those in *cyclo*hexane (Part I, Table I, p. 221).

I am indebted to Miss I. Thompson for technical assistance and to Mr. R. McWilliam for carrying out the chlorine-36 griseofulvin fermentations.

REFERENCES

1. MacMillan, J., *Chem. & Ind.*, 1951, 719.
2. ——, *J. Chem. Soc.*, 1953, 1697.
3. British Patent Application No. 9012/55.
4. Grove, J. F., MacMillan, J., Mulholland, T. P. C., and Rogers, M. A. T., *J. Chem. Soc.*, 1952, 3949.
5. Rodin, N. S., *Nucleonics*, 1947, **1**, 48.
6. Ashton, G. C., and Foster, M. C., *Analyst*, 1955, **80**, 123.
7. Brian, P. W., Curtis, P. J., and Hemming, H. G., *Trans. Brit. Mycol. Soc.*, 1946, **29**, 173.

GLAXO LABORATORIES LTD.
SEFTON PARK
STOKE POGES, BUCKS.

July 27th, 1955

(40)

A Method of Correcting for Irrelevant Absorption in Ultra-violet Spectrophotometric Analysis

BY

G. C. ASHTON and J. P. R. TOOTILL

Reprinted from THE ANALYST, *the Journal of the Society for Analytical Chemistry, April*, 1956, *Vol.* 81, *No.* 961, *pp.* 232–238

A Method of Correcting for Irrelevant Absorption in Ultra-violet Spectrophotometric Analysis

By G. C. ASHTON and J. P. R. TOOTILL

A method of correcting for impurities having quadratic absorption spectra in samples for ultra-violet spectrophotometric analysis, described previously as part of a spectrophotometric assay for griseofulvin, has been applied to experimental mixtures of griseofulvin and materials showing various types of irrelevant absorption. The previously described correction procedure is shown to be highly satisfactory for such irrelevant absorption.

The extension of the correction procedure to other types of irrelevant absorption and to mixtures of substances absorbing in the ultra-violet region is discussed. The effect of this type of correction procedure on the precision of the estimate and a method for determining the degree of curvature of the irrelevant-absorption spectrum are described in Appendixes.

The determination of a substance by measuring its absorption in the ultra-violet region is much-practised. When the spectrum shows a convenient peak or peaks, *i.e.*, regions of sharply defined maximum absorption, measurement of the extinction value at the point of maximum absorption will provide a precise and accurate measure of the substance, providing it is pure. However, it is unusual to deal with pure substances in analysis; more often than not use of an ultra-violet spectrophotometric method is difficult or impossible, because of the presence of irrelevant absorption in the same ultra-violet region, not characteristic of the substance being analysed but due to impurities remaining after preparation of the spectrophotometric sample. The nature of the irrelevant absorption is usually not known, although the plot against wavelength of the curve for irrelevant absorption can sometimes be determined; it is often found to be a reasonably smooth curve, approximating to a quadratic.

Two approaches are then possible. One is to find a way to remove the substances causing the irrelevant absorption, while still giving a quantitative isolation of the substance being assayed. This can sometimes be done by washing with suitable solvents, by chromatography or by other fractionation procedures. It is unusual to be able to remove the source of interference completely. The second approach is to establish a method of correcting for the irrelevant absorption by a mathematical device. Even with a suitable correction procedure, it is still advisable to keep the irrelevant absorption to the minimum.

One of the most widely used procedures for correcting for irrelevant absorption is that due to Morton and Stubbs,[1] which has been widely described and discussed[2,3,4] and does not require description here. Its fundamental requirement is that a series of wavelengths can be found (three are usually chosen) such that the irrelevant absorption can be joined by a straight line. Shaw[4] has developed an extension of the Morton and Stubbs procedure to correct for irrelevant absorption in solutions of ergosterol extracted from yeasts. His procedure also requires that the irrelevant absorption be linear through the fixation points.

It is obvious that with irrelevant absorption following a quadratic curve, the correction procedure of Morton and Stubbs cannot be applied. For purposes of this paper quadratic curvature is defined as meaning that the relation between absorption and wavelength can be adequately described by a quadratic equation, in the sense that higher terms such as cubic, quartic, etc., if present, are of too small magnitude to affect significantly the estimate of potency. In our experience, this is the most usual type of irrelevant absorption outside the vitamin-A field.

To overcome the limitations of the Morton and Stubbs procedure, we have described a correction procedure for impurities giving quadratic absorption spectra in butyl acetate extracts of griseofulvin.[5] It is based on the development of a series of coefficients highly correlated with the shape of the griseofulvin spectrum in the region of the maximum, but statistically independent of uniform, linear and quadratic absorption spectra.

The advantages of this procedure are—

(*a*) The experimental determination in the irrelevant absorption of artificial "fixation points" that can be joined by a straight line is not required.

(*b*) Provided that the curve for irrelevant absorption can be adequately expressed by an equation not more complex than quadratic, its absolute value and position is not critical.

We have found with griseofulvin solvent extracts of varying origin that the underlying absorption curve is never more complex than a simple quadratic. The precise degree of curvature, the position of the peak and the amount and nature of the irrelevant absorbing material vary considerably from extract to extract. The recommended correction procedure is, however, of general application to these extracts and hence of particular value in routine analysis.

(*c*) Although we have found only quadratic irrelevant-absorption spectra, the method of correlated correction coefficients could be extended, if necessary, to more complex forms of irrelevant absorption, *e.g.*, cubic. The limitation of the method is that forms of irrelevant absorption of complexity equal to or greater than that of the absorption spectrum of the substance being assayed cannot be unequivocally distinguished unless the precise shape of the irrelevant-absorption spectrum is known (see "Discussion" below).

Any number of wavelengths can be used in excess of three, but we have chosen seven. In this way the precision of the corrected estimate when dealing with sharp maxima is considerably improved (see Appendix I).

The calculation of these coefficients and other information about the procedure have been reported previously.[5] This paper describes results obtained by applying the correction to samples of griseofulvin to which have been added artificial sources of irrelevant absorptions.

Experimental

The correction procedure was evaluated by adding ultra-violet absorbing substances to solutions of pure griseofulvin in butyl acetate. The reagents chosen to act as irrelevant absorption had to fulfil the following conditions—

(*a*) they must be sufficiently soluble in butyl acetate to give an absorbance of about 0·7 at 290 mμ in a 1-cm cell,
(*b*) their absorption spectra in butyl acetate solution must be stable over the range 280 to 300 mμ for at least the time required to carry out the experiment,
(*c*) they should not interact with griseofulvin, and
(*d*) they must have absorption spectra of the desired type over the range 280 to 300 mμ.

Of the many substances examined the following were chosen as suitable for our purposes—

(*i*) *p*-nitrophenylacetic acid, which has a nearly linear absorption spectrum (see Table I),
(*ii*) 6-methylquinoline, with an absorption spectrum approximating to a simple curve, in the sense of the definition employed in this paper,
(*iii*) 2-aminoquinoline, which has a markedly curved absorption spectrum with a minimum at 292 to 293 mμ, and
(*iv*) anthrone, which has a more complex absorption spectrum with a small peak at approximately 296 mμ.

Table I

Absorbances of griseofulvin and irrelevant-absorption substances in butyl acetate (mean of two replicates)

Substance	Wavelength						
	288 mμ	290 mμ	292 mμ	294 mμ	296 mμ	298 mμ	300 mμ
Griseofulvin	0·658	0·656	0·559	0·431	0·301	0·209	0·161
p-Nitrophenylacetic acid	0·739	0·660	0·578	0·510	0·441	0·373	0·317
6-Methylquinoline ..	0·639	0·605	0·582	0·545	0·515	0·500	0·464
2-Aminoquinoline ..	0·718	0·641	0·618	0·634	0·680	0·759	0·860
Anthrone..	0·697	0·655	0·634	0·639	0·641	0·618	0·568

p-Nitrophenylacetic acid and 6-methylquinoline have to different degrees the type of quadratic irrelevant-absorption spectra commonly found in quantitative ultra-violet spectrophotometry: 2-aminoquinoline represents a type of curved absorption spectrum that at first sight might appear quadratic, but is shown below to be of more complex nature, whereas anthrone represents a type of curved absorption spectrum quite clearly of more complex nature, having in addition to its general curvature a slight peak in the neighbourhood of the griseofulvin maximum.

PREPARATION OF SOLUTIONS—

In each experiment the material showing irrelevant absorption was added at four levels. For convenience the levels are described as "percentage impurity" and were usually 10, 25, 50 and 75 per cent.

The "percentage-impurity" figures describe the proportions in which the original griseofulvin solution in butyl acetate is mixed with the irrelevant-absorption butyl acetate solution, *e.g.*, 25 per cent. impurity is three parts of griseofulvin solution and one part by volume of irrelevant-absorption solution.

The griseofulvin solution was prepared freshly for each irrelevant-absorption substance by dissolving 9 to 10 mg of purified griseofulvin in 1 litre of butyl acetate. The actual concentration was determined by the seven-point correction procedure by means of coefficients that had been determined previously for the sample of griseofulvin. These solutions of griseofulvin had absorbances between 0·6 and 0·7.

The irrelevant-absorption solutions were prepared by dissolving sufficient of the appropriate substance in butyl acetate to give an absorbance of between 0·6 and 0·7 at 290 mμ.

The various dilutions were prepared in 100-ml grade-A calibrated flasks with grade-A pipettes, by taking the appropriate volume of each solution.

Separate solutions and dilutions were prepared for each experiment, each griseofulvin master solution being used for preparing only one set of irrelevant-absorption dilutions.

TABLE II

RESULTS OBTAINED AT 288 TO 300 mμ

Irrelevant absorption	Percentage of irrelevant absorption	Observed results	Theoretical results
p-Nitrophenylacetic acid	0	9·08, 9·08	9·08
	15	7·41, 7·65	7·71
	25	6·80, 6·80	6·80
	50	4·53, 4·44	4·49
	75	2·21, 2·30	2·25
	100	0·07, 0·15	Nil
6-Methylquinoline	0	9·47, 9·55	9·51
	10	8·70, 8·79	8·56
	25	7·17, 6·96	7·13
	50	4·91, 5·02	4·75
	75	2·82, 2·50	2·38
	100	0·05, 0·12	Nil
2-Aminoquinoline	0	9·11, 9·12	9·11
	10	8·09, 8·13	8·20
	25	6·36, 6·79	6·84
	50	3·79, 3·75	4·56
	75	0·83, 0·85	2·28
	100	− 1·65, − 1·74	Nil
Anthrone	0	9·27, 9·31	9·29
	10	7·98, 8·08	8·36
	25	6·10, 6·15	6·97
	50	7·37, 7·45	4·65
	75	5·50, 5·33	2·32
	100	2·58, 2·61	Nil

NOTE—Figures represent μg of griseofulvin per ml in solution.

DETERMINATION OF ABSORBANCES—

The spectrophotometric measurements were made on a Unicam SP500 quartz spectrophotometer. Each dilution was measured at the seven wavelengths in two separate 1-cm silica cells against butyl acetate, thus giving a measure of the precision of the method.

Measurements were made at seven wavelengths, 288 to 300 mμ, at intervals of 2 mμ.

Results

The results obtained are shown in Table II.

From Table II it will be seen that the experimental results with *p*-nitrophenylacetic acid and 6-methylquinoline are in good agreement with the theoretical results irrespective of the level of irrelevant absorption.

With 2-aminoquinoline the results obtained are markedly discrepant at the 50 per cent. impurity level. For anthrone the discrepancy increases with increasing levels of impurity. With both the existence of an appreciable level of "apparent griseofulvin," negative or positive, in the solution containing no griseofulvin indicates that both substances have more complex absorption spectra than can be approximated by a quadratic. With *p*-nitrophenylacetic acid and 6-methylquinoline (Table II) the solutions containing no griseofulvin give (within experimental error) the correct answer, namely zero, and this establishes that the coefficients employed are in fact orthogonal to their absorption spectra. In other words, the absorption spectra of these substances can be adequately represented by a quadratic, as required for this particular correction procedure.

These results show that impurities with quadratic irrelevant-absorption spectra can be accurately accounted for by the correction procedure, even when the amount of irrelevant absorption is two or three times that due to the substance being assayed.

Discussion

The correction procedure as described[5] is capable of dealing with impurities having quadratic absorption spectra. However, this type of correction can be extended to more complex cases in two ways. These are as follows—

(1) When the irrelevant-absorption spectrum is more complex than a quadratic, though still of lower order than that of the substance being assayed, the principles of the correction remain the same and involve constructing a set of coefficients highly correlated with the absorption spectrum of the substance being assayed, but statistically independent over the wavelengths employed of linear, quadratic, cubic and so on, spectra as far as is required. Full details of the manner of establishing the order of complexity required to define the irrelevant-absorption spectrum are given in Appendix II.

(2) When the irrelevant absorption is known to be due to a substance, or more than one, whose absorption spectra are accurately known, in which event, coefficients are calculated that over the wavelengths employed are statistically independent of the irrelevant-absorption spectrum, but are most highly correlated with the absorption spectrum of the substance being assayed. An example of this approach applied to the 2-aminoquinoline and anthrone mixtures with griseofulvin (previously shown in Table II) is given in Table III.

Table III

Results from Table II recalculated by method two outlined in the discussion

Irrelevant absorption	Percentage of irrelevant absorption	Recalculated results	Theoretical results
2-Aminoquinoline	0	9·12, 9·11	9·11
	10	8·21, 8·21	8·20
	25	6·87, 6·88	6·84
	50	4·53, 4·52	4·56
	75	2·26, 2·28	2·28
	100	0·57, − 0·57	Nil
Anthrone	0	9·30, 9·28	9·29
	10	8·35, 8·38	8·36
	25	6·96, 6·99	6·97
	50	5·27, 5·30	4·65
	75	2·15, 2·20	2·32
	100	0·13, − 0·13	Nil

With 2-aminoquinoline the results of this alternative correction procedure are highly satisfactory and show that a simple additive relationshp holds between the absorbances of the constituents.

With anthrone, however, agreement, though improved, is not complete. Although we have not studied this phenomenon further, the results suggest that the absorbances are not strictly additive, some interaction occurring.

By extension of this principle, the simultaneous assay of non-interacting, binary, tertiary, etc., mixtures of substances with characteristic ultra-violet absorption is possible even when the peaks of the substances concerned are not sufficiently separated to permit their separate assay by their respective peaks.

We acknowledge with thanks the technical assistance of Miss I. Thompson.

APPENDIX I

LOSS OF PRECISION WITH INCREASING COMPLEXITY OF CORRECTIONS—

The absorbance of the sample at any given wavelength, λ, is assumed to be the sum of the absorbance of the griseofulvin in the sample and irrelevant absorbance of the impurity, the latter being given by an n^{th} degree polynomial function in λ, whose constants are to be determined from the internal evidence of the assay.

At the given wavelength λ let—
S = absorbance of pure standard griseofulvin solution,
U = absorbance of sample,
p = ratio of griseofulvin in sample compared with the standard,
x_m = the value of a polynomial of m^{th} degree in λ, $m \leqslant n$, and
a_m = constant defining the m^{th} polynomial of irrelevant absorption.

Then—

$$U = p\,S + a_0 x_0 + a_1 x_1 + a_2 x_2 \ldots + a_m x_m.$$

For convenience the x polynomials are orthogonal and are defined as follows, summation being over all wavelengths employed in the assay—

$$\Sigma\, x_{m_1} x_{m_2} = 0,\ m_1 \neq m_2$$
$$= 1,\ m_1 = m_2$$

The normal equations, in matrix form, are then as follows—

$$\begin{bmatrix} \Sigma S^2 & \Sigma S x_0 & \Sigma S x_1 & \Sigma S x_2 \ldots & \Sigma S x_n \\ \Sigma S x_0 & 1 & 0 & 0 \ldots & 0 \\ \Sigma S x_1 & 0 & 1 & 0 \ldots & 0 \\ \Sigma S x_2 & 0 & 0 & 1 \ldots & 0 \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ \Sigma S x_n & 0 & 0 & 0 \ldots & 1 \end{bmatrix} \begin{bmatrix} p \\ a_0 \\ a_1 \\ a_2 \\ \cdot \\ \cdot \\ \cdot \\ a_n \end{bmatrix} = \begin{bmatrix} \Sigma SU \\ \Sigma U x_0 \\ \Sigma U x_1 \\ \Sigma U x_2 \\ \cdot \\ \cdot \\ \cdot \\ \Sigma U x_n \end{bmatrix}$$

The solution of the normal equations by inversion of the matrix is thus—

$$\frac{1}{D}\begin{bmatrix} 1 & -\Sigma S x_0 & -\Sigma S x_1 & -\Sigma S x_2 \ldots & -\Sigma S x_n \\ -\Sigma S x_0 & D+(\Sigma S x_0)^2 & \Sigma S x_0 \Sigma S x_1 & \Sigma S x_0 \Sigma S x_2 \ldots & \Sigma S x_0 \Sigma S x_n \\ -\Sigma S x_1 & \Sigma S x_0 \Sigma S x_1 & D+(\Sigma S x_1)^2 & \Sigma S x_1 \Sigma S x_2 \ldots & \Sigma S x_1 \Sigma S x_n \\ -\Sigma S x_2 & \Sigma S x_0 \Sigma S x_2 & \Sigma S x_1 \Sigma S x_2 & D+(\Sigma S x_2)^2 \ldots & \Sigma S x_2 \Sigma S x_n \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ -\Sigma S x_n & \Sigma S x_0 \Sigma S x_n & \Sigma S x_1 \Sigma S x_n & \Sigma S x_2 \Sigma S x_n \ldots & D+(\Sigma S x_n)^2 \end{bmatrix} \begin{bmatrix} \Sigma SU \\ \Sigma U x_0 \\ \Sigma U x_1 \\ \Sigma U x_2 \\ \cdot \\ \cdot \\ \cdot \\ \Sigma U x_n \end{bmatrix} = \begin{bmatrix} p \\ a_0 \\ a_1 \\ a_2 \\ \cdot \\ \cdot \\ \cdot \\ a_n \end{bmatrix}$$

where $D = \Sigma S^2 - (\Sigma S x_0)^2 - (\Sigma S x_1)^2 - (\Sigma S x_2)^2 \ldots - (\Sigma S x_n)^2$.

Now the variance of p, the estimate of griseofulvin concentration in the sample, is proportional to $1/D$.

From this relationship the following facts can be deduced—

(1) In general a loss of precision will occur for each additional term of the polynomial required to describe the irrelevant-absorption spectrum.

(2) Hence the number of terms employed should not be excessive and should not be taken to the point where gain in accuracy is more than offset by loss in precision.

(3) If the complexity of the irrelevant-absorption spectrum is of the same order as that of the griseofulvin curve, say of m^{th} degree, so that the griseofulvin curve could be defined by an m^{th} degree polynomial, then D will be zero and the method fails. Clearly the method will also fail even when the irrelevant-absorption spectrum is not particularly complex, say of order m, if less wavelength settings than $m + 2$ are employed.

(4) In view of the loss of precision with additional correction terms, an increased number of absorbance readings is required to reduce the error of the method. This may be done either by replicatory readings at the minimum number of wavelength settings dictated by the complexity of the correction or by reading at more wavelength settings.

APPENDIX II

DETERMINATION OF THE DEGREE OF THE IRRELEVANT ABSORPTION—

The orthogonal polynomials x_0, x_1, x_2, etc., employed in Appendix I, though orthogonal to each other, are not orthogonal to the griseofulvin curve. To ascertain which of these components is introducing significant bias into the results and to determine the magnitude of the bias introduced, there is a considerable advantage in using functions mutually orthogonal and orthogonal to the griseofulvin-absorption spectrum over the wavelengths employed. These functions are designed to estimate successively the uncorrect griseofulvin estimate, correction for uniform absorption, additional correction for linear absorption and so on.

With the same nomenclature as in Appendix I, the following functions have the desired properties—

$$S$$

$$S - x_0 \frac{\Sigma S^2}{\Sigma S x_0}$$

$$S - x_0 \Sigma S x_0 - x_1 \left\{ \frac{\Sigma S^2 - (\Sigma S x_0)^2}{\Sigma S x_1} \right\}$$

$$S - x_0 \Sigma S x_0 - x_1 \Sigma S x_1 - x_2 \left\{ \frac{\Sigma S^2 - (\Sigma S x_0)^2 - (\Sigma S x_1)^2}{\Sigma S x_2} \right\}$$

$$S - x_0 \Sigma S x_0 - x_1 \Sigma S x_1 - x_2 \Sigma S x_2 - x_3 \left\{ \frac{\Sigma S^2 - (\Sigma S x_0)^2 - (\Sigma S x_1)^2 - (\Sigma S x_2)^2}{\Sigma S x_3} \right\}$$

and so on.

In practice the coefficients corresponding to these functions would not be evaluated. The various sums involved ΣS^2, $\Sigma S x_0$, $\Sigma S x_1$, $\Sigma S x_2$, etc., and the corresponding sums ΣSU, $\Sigma U x_1$, $\Sigma U x_2$, $\Sigma U x_3$, etc., would be evaluated from the known values of S and U and from suitable whole number multiples of x_0, x_1, x_2, etc.,[6] and these sums entered into the following formulae derived from the aforementioned functions.

Let p = uncorrected griseofulvin estimate,
let Δp_0 = correction to be added for irrelevant uniform absorption,
let Δp_1 = correction to be added for irrelevant linear absorption,
let Δp_2 = correction to be added for irrelevant quadratic absorption,
let Δp_3 = correction to be added for irrelevant cubic absorption,
etc.

Then—

$$p = \frac{\Sigma SU}{\Sigma S^2}$$

$$\Delta p_0 = \frac{(\Sigma Sx_0)^2 \left\{ \Sigma SU - \frac{\Sigma Ux_0 \, \Sigma S^2}{\Sigma Sx_0} \right\}}{\Sigma S^2 \{\Sigma S^2 - (\Sigma Sx_0)^2\}}$$

$$\Delta p_1 = \frac{(\Sigma Sx_1)^2 \left[\Sigma SU - \Sigma Ux_0 \, \Sigma Sx_0 - \frac{\Sigma Ux_1}{\Sigma Sx_1} \left\{ \Sigma S^2 - (\Sigma Sx_0)^2 \right\} \right]}{\{\Sigma S^2 - (\Sigma Sx_0)^2\}\{\Sigma S^2 - (\Sigma Sx_0)^2 - (\Sigma Sx_1)^2\}}$$

$$\Delta p_2 = \frac{(\Sigma Sx_2)^2 \left[\Sigma SU - \Sigma Ux_0 \, \Sigma Sx_0 - \Sigma Ux_1 \, \Sigma Sx_1 - \frac{\Sigma Ux_2}{\Sigma Sx_2} \left\{ \Sigma S^2 - (\Sigma Sx_0)^2 - (\Sigma Sx_1)^2 \right\} \right]}{\{\Sigma S^2 - (\Sigma Sx_0)^2 - (\Sigma Sx_1)^2\} \{\Sigma S^2 - (\Sigma Sx_0)^2 - (\Sigma Sx_1)^2 - (\Sigma Sx_2)^2\}}$$

etc.

And the sum of squares attributable to each correction may be expressed as follows—

for p : $p\Sigma SU$

for Δp_0: $\Delta p_0 \left[\Sigma SU - \frac{\Sigma Ux_0 \, \Sigma S^2}{\Sigma Sx_0} \right]$

for Δp_1: $\Delta p_1 \left[\Sigma SU - \Sigma Ux_0 \, \Sigma Sx_0 - \frac{\Sigma Ux_1}{\Sigma Sx_1} \left\{ \Sigma S^2 - (\Sigma Sx_0)^2 \right\} \right]$

for Δp_2: $\Delta p_2 \left[\Sigma SU - \Sigma Ux_0 \, \Sigma Sx_0 - \Sigma Ux_1 \, \Sigma Sx_1 - \frac{\Sigma Ux_2}{\Sigma Sx_2} \left\{ \Sigma S^2 - (\Sigma Sx_0)^2 - (\Sigma Sx_1)^2 \right\} \right]$

etc.

In this manner an analysis of variance, centred at zero and not at the mean as usual, may be performed, in which the total variation in the absorbances recorded for the sample may be subdivided into categories attributable to the uncorrected potency estimate, correction for uniform absorption, correction for linear absorption, correction for quadratic absorption and so on.

The variance attributable to each source may be compared with the random error in the readings, and only those corrections that are significant need be considered as introducing significant bias into the result. Such an analysis is summarised in our previous paper.[5]

This process carried out on a suitably representative selection of the samples concerned will indicate the order of correction required. Coefficients suitable for routine use may then be introduced.[5]

References

1. Morton, R. A., and Stubbs, A. L., *Analyst*, 1946, **71**, 348.
2. Gridgeman, N. T., *Ibid.*, 1951, **76**, 449.
3. Bagnall, H. H., and Stock, F. G., *J. Pharm. Pharmacol.*, 1952, **4**, 81.
4. Shaw, W. H. C., and Jefferies, J. P., *Analyst*, 1953, **78**, 519.
5. Ashton, G. C., and Tootill, J. P. R., *Ibid.*, 1956, **81**, 225.
6. Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," Fourth Edition, Oliver & Boyd, Edinburgh, 1953, p. 80 *et seq.*

Glaxo Laboratories Ltd.
Sefton Park
Stoke Poges, Bucks.

September 26th, 1955

Reprinted from THE ANALYST, *the Journal of the Society for Analytical Chemistry,*
July, 1956, *Vol.* 81, *No.* 964, *pp.* 433–442

(41)

Symposium on the Use of Radioactive Materials in Biological Assay

AT a Joint Meeting of the Biological Methods Group, the Physical Methods Group and the Scottish Section with the Edinburgh and East of Scotland Sections of the Royal Institute of Chemistry, the Society of Chemical Industry and the Chemical Society held on Monday and Tuesday, July 11th and 12th, 1955, in the Department of Biochemistry, University New Buildings, Edinburgh, 8, nine papers were presented and discussed under the chairmanship of Professor G. F. Marrian, F.R.S., on July 11th and of Dr. L. J. Harris, Chairman of the Biological Methods Group, on July 12th. The papers and discussions are summarised below.

THE DETERMINATION OF RADIOACTIVE ISOTOPES IN BIOLOGICAL SAMPLES

DR. R. F. GLASCOCK began by asking the forgiveness of those who were experienced in the use of radioactive isotopes, because the first part of his paper was on a somewhat elementary level. His paper was to be regarded as introductory to the more specialised papers that were to follow on the applications of radioactive isotopes.

He then outlined the general properties of radioactive substances, referring briefly to radiations and half-lives. In general, he said, beta-ray emitters were most convenient for biochemical work because no special shielding of the worker was necessary even when high activities were being handled. Gamma-ray emitters were nevertheless of great use in studies of the distribution of chemicals in the living animal because the presence of the labelled material could then be detected from outside the body. A useful example of such an isotope was iodine-131. Half-lives varied from fractions of a second to millions of years, those at the extremes being of least practical use as tracers—particularly, for obvious reasons, the very short-lived ones. Very long-lived isotopes suffered from the disadvantage that high specific activities were not obtainable. Thus carbon-14, having a half-life of about 5000 years, would have been even more useful than it was if its half-life had been much shorter. For work on the tracing of substances that were physiologically active at very low doses, carbon-14 could not confer a sufficiently high specific activity. For this purpose tritium, having a half-life of 11 years, was much more suitable: higher specific activities were available, yet the isotope did not decay appreciably during the course of an ordinary experiment. Tritium, of course, had its disadvantages, chiefly that the beta-particles were of very low energy.

The speaker then discussed instruments of detection, the most important being the Geiger counter. Various patterns available commercially were illustrated. The ordinary end-window counter, the thickness of the window depending on the energy of the beta-particles to be detected, was the most widely used instrument for the assay of solid samples, whilst the liquid counter was suitable for assaying isotopes in solution emitting particles energetic enough to penetrate both the solution and the relatively thick glass wall. End-window counters filled with helium at atmospheric pressure and with windows less than 2 mg per sq. cm in thickness were suitable for assaying carbon-14 and had a total efficiency of about 3 per cent. for this isotope. Liquid counters, on the other hand, were suitable only for isotopes, such as phosphorus-32, emitting fairly penetrating radiations, and even then their efficiency was only about one-twentieth of that of the end-window counter. Such counters varied among themselves, however, owing to the fact that the thin glass area or "window" encasing the sensitive zone was hand-made and the thickness varied.

The speaker next discussed the chemical procedures that were necessary before counting. Solid untreated samples of fat, protein, tissues or other material could be counted direct beneath an end-window counter, but a source of inaccuracy in such work was uncertainty about the proportion of radioactive element in the sample. It was better to reduce the radioactive element to some standard chemical form, metals being precipitated as their insoluble salts, sulphur being converted to barium sulphate, phosphorus to phosphate, carbon to carbon dioxide or barium carbonate and so on. All samples within an experiment were then strictly comparable. If they were not reduced to a common chemical form, the counting rate could be corrected to a common basis from a knowledge of the percentage composition of the sample and the back-scattering factor of the elements contained in it.

An account was finally given of gas-counting procedures for carbon and hydrogen isotopes as used in the Isotope Section of the National Institute for Research in Dairying. These in general were much more sensitive and accurate than any other method, although they were slower. Speed was not an important consideration, because the time occupied by counting in most experiments was only a small proportion of the total time spent, but the greater sensitivity permitted a reduction to a tenth of the total amount of isotope used.

The general principle of the technique was the combustion of 10-mg samples, separation of the water and carbon dioxide produced and conversion of water to butane by reaction with dry butyl magnesium bromide. Carbon dioxide and butane were then made part of the filling of Geiger counters for the determination of carbon-14 and tritium, respectively. If carbon-13 was also present, part of the carbon dioxide was set aside for mass-spectrometric analysis. Full details of the technique had already been published (R. F. Glascock, "Isotope Gas Analysis for Biochemists," Academic Press Inc., New York and London, 1954).

Discussion

A MEMBER of the audience asked the author for his opinion on the relative merits of methane and helium plus methane for proportional counting.

DR. GLASCOCK said that if methane was used the counters had to be operated in the proportional region, whereas if helium mixed with a suitable quenching vapour was used, the counters could be operated in the Geiger region. The question thus resolved itself into a matter of personal choice: whether the simpler gas line and the more complicated electronic equipment for proportional counting suited the worker better than more complicated filling apparatus (for the introduction of the quenching vapour) and less complicated electronic equipment An additional factor was the cost of the helium, which was considerably greater than that of methane.

DR. A. R. SOMERVILLE commented that helium cost about 21s. per cubic foot, and that it was soon more economical to buy the extra electronic equipment for proportional counting. He said that they had tried using butane in the Geiger regions, but that the operating voltage at atmospheric pressure was over 4 kV.

MR. F. P. W. WINTERINGHAM pointed out that, although helium was expensive, there was no need to use it invariably by a "flow-counting" technique; it was much more economical to evacuate the tube and then fill it from a suitable high-vacuum manifold for each count.

DR. WATKINS enquired whether the methane used in a gas-flow counter had to be of high purity and whether, for instance, the presence of traces of oxygen in the gas would lead to spurious counts.

DR. GLASCOCK said he had no direct experience of methane proportional counters, but he believed that they were less sensitive to impurities than were Geiger counters. D. F. White, I. G. Campbell and P. R. Payne (*Nature*, 1950, **166**, 628), for example, in their tritium-assay method, had reported that methane formed by the action of water on Al_4C_3 could be used in a proportional counter but not in a Geiger counter, and they attributed the absence of a Geiger plateau to impurities in the gas.

MR. G. C. ASHTON asked if the author had ever noticed retention of radioactivity after burning carbon-14 compounds, either in the fillings or walls of the combustion tube, and if any difficulties had been experienced owing to atmospheric carbon dioxide leaking into the combustion apparatus.

DR. GLASCOCK replied that Mr. Ashton was evidently referring to "memory effects," which had never been noticed with carbon-14 compounds; with care, these could be avoided even with tritium compounds. As for atmospheric carbon dioxide leaking into the apparatus, there was no danger of this if the vacuum line was made completely leak-proof, and the speaker referred to Dr. McFarlane for confirmation, as he knew that Dr. McFarlane also had operated a gas-counting apparatus.

DR. A. S. MCFARLANE said that they had had no trouble from contamination of the combustion tube in some thousands of combustions. As to the relative merits of the various gas diluents and quenching agents mentioned by Dr. Glascock, he pointed out that, provided a 3-kV power pack and pre-amplifier were available for proportional counting, it was not in their experience necessary or advantageous to use any gas other than the carbon dioxide.

MR. F. P. W. WINTERINGHAM, in a discussion of the relative merits of liquid and end-window counters, said that his experience with liquid beta counters largely confirmed the relative sensitivities to phosphorus-32 and iodine-131 that had been published. He suggested that Korff's recommended use of the terms "efficiency" and "sensitivity" should be used more rigorously, the former referring to response to ionising events within the sensitive volume of the tube and the latter to the effective response of the tube to the sample (S. A. Korff, "Electron and Nuclear Counters," D. Van Nostrand Co. Inc., New York, 1946, p. 16).

DR. W. F. R. POVER said that they had used liquid counters extensively with potassium-42, phosphorus-32 and iodine-131. The measured "counter factor," *i.e.*, the number of counts per second given by the tube divided by the total number of disintegrations per second of the isotope in the tube, was found to be between 8 and 10 per cent. for M6 counters, when using an N.P.L. standard phosphorus-32 source

The "counter factor" varied from tube to tube, but was in the range claimed by the manufacturers (20th Century Electronics) and was comparable with end-window "counter factors" and was not, as suggested by Dr. Glascock, considerably lower. With N.P.L. iodine-131 standards, they found counter factors of 0·5 to 1·0 per cent. On the question of contamination or "memory" effect of liquid counters, Dr. Pover commented that, although an acid phosphate wash was very effective, the real answer was to use ample amounts of carrier in the same molecular form as the active isotope. They had noticed the effect with iodine-131-labelled fat, but it was considerably reduced by using inactive-iodine-labelled fat carrier. To avoid density corrections when liquid-counting fat-soluble substances, he said they used a mixture of about 3 parts of toluene to 1 part of chloroform made up to the density of water; this mixture was an excellent fat solvent. Dr. Pover asked Dr. Glascock and the meeting in general whether they had any experience of the ionisation-chamber method of radioactive assay with carbon-14 and tritium.

DR. GLASCOCK said that he had no experience of ionisation chambers, but he had gathered from what he had read and from information he had got from people who had used them that they were not very suitable for routine work.

DR. E. LESTER SMITH pointed out that although the solution counter might give less counts than the end-window counter for a given amount of sample, it scored heavily in other ways. Usually a much larger amount could be introduced into the sensitive volume, so that a higher actual counting rate could be secured, provided sufficient material was available. The trouble of plating out was avoided and the sample did not need to be purified. It was possible, for example, to count wet digests of organs directly in concentrated acid or alkaline solutions. Memory effects could usually be prevented by adding inactive carrier to the sample.

DR. N. MILLER said that they had recently had occasion to calibrate two M6 liquid counters, using standard phosphorus-32 solutions from the National Physical Laboratory. Both counters recorded about 7 per cent. of the disintegrations taking place within the 10-ml volume of solution used. The phosphorus-32 was made up in 0·8 *N* sulphuric acid before assay and no trouble was encountered with memory effects even when carrier-free material was used. With carrier-free material, four washings with distilled water sufficed to return the counting rate to its background value.

DR. J. F. TAIT referred to an earlier question by Mr. Ashton on memory effects, and said that they had not found gas counting to be subject to memory effects with carbon-14 dioxide or even with tritium gas. Although gas counting was the more general method, it could be expected that the ratio of efficiency to background of liquid scintillation counters would approach that of gas-counting methods. Whilst the difficulties of assaying impure materials with the liquid scintillation counter were not so likely to be overcome, this method was more suitable for routine assays of pure specimens.

THE PRINCIPLES OF ISOTOPE-DILUTION ASSAY WITH SPECIAL REFERENCE TO VITAMIN B_{12}

DR. E. LESTER SMITH said that the principles of isotope-dilution assay were simple, though in practice complications often arose. The substance to be determined must first be prepared labelled with a radioactive (or stable) isotope. A small known amount of this "marker" was then added to the sample, and the substance was isolated in a pure state. The yield could be low, because the diminution in radioactivity during isolation provided an exact correction factor. The technique was useful as an "absolute" method, capable of discriminating between isomers or related compounds. Trenner and his colleagues (N. R. Trenner, R. W. Walker, B. Arison and C. Trumbauer, *Anal. Chem.*, 1951, **23**, 487; N. R. Trenner, R. W. Walker, B. Arison and R. P. Buhs, *Anal. Chem.*, 1949, **21**, 285) had, for example, used deuterium-labelled markers for the determination of nicotinic acid and the gamma isomer of hexachlor*ocyclo*hexane; the deuterium contents of the products were determined from specific infra-red absorption bands. Isotope-dilution assays were also valuable when the substance exhibited no specific reactions, or none that could be exploited with crude samples.

Vitamin B_{12} could be determined in fermentation liquors and concentrates with a marker biosynthesised by fermentation in presence of cobalt-60. The size of the sample needed and the numerous steps required to achieve adequate purity of the isolate made the method somewhat laborious. F. A. Bacher, A. E. Boley and C. E. Shunk (*Anal. Chem.*, 1954, **26**, 1146) removed impurities with a zinc salt and alkali and applied one or more solvent-extraction steps and then chromatographed over two ion-exchange resins. The author preferred paper chromatography for the final step, because it removed possible degradation products of vitamin B_{12} and analogues of the vitamin, besides certain other impurities. Either actual or potential cyanocobalamin could be determined, by omitting or including, respectively, treatment with nitrite and cyanide, which converted other cobalamins into cyanobalamin.

When the substance happened to be tagged already, the accurate micro method of inverse isotope-dilution assay could be applied. A large excess of the unlabelled substance was added as "carrier" so that its re-isolation was relatively easy. The same advantages accrued with the related isotope-derivative method, which should be widely applicable, *e.g.*, in the steroid field.

The method was pioneered by Keston and co-workers (A. S. Keston, S. Udenfriend and S. K. Cannan, *J. Amer. Chem. Soc.*, 1949, **71**, 249; A. S. Keston, S. Udenfriend and M. Levy, *J. Amer. Chem. Soc.*, 1950, **72**, 748) for the determination of selected amino acids. Small samples of protein hydrolysate were treated quantitatively with [^{131}I]*p*-iodophenylsulphonyl chloride ("pipsyl" chloride). The "pipsyl" derivatives were extracted with ether and mixed with excess of the unlabelled "pipsyl"-amino acid to be determined; the compound was re-isolated and purified by repeated crystallisation and charcoal treatment to constant specific activity. Partial racemisation during hydrolysis introduced no errors, since racemic carrier could be used; alternatively, optical isomers could be determined specifically with the appropriate carrier. Difficulties in removing radioactive impurities, such as "pipsyl"-amide, traces of "dipipsyl" derivatives and especially other co-precipitated "pipsyl"-amino acids, tended to outweigh the essential simplicity and elegance of this approach.

Purification by paper chromatography was effective, but was best exploited by the isotopic-indicator method. A trace amount of the [^{35}S]"pipsyl"-amino acid was added and the eluate from the appropriate chromatographic zone was counted with and without an aluminium screen, to determine separately the radiation from the iodine-131 and sulphur-35 markers. The ratio of these activities was involved in the calculations.

Discussion

Dr. W. Mulligan pointed out that, in determining sulphur-35 and iodine-131 in the same sample by screening off the radiations from the sulphur-35, it was of value to confirm the result by allowing the iodine-131 to decay completely and determining the sulphur-35. This was possible because of the difference in half-lives of the two isotopes.

Dr. A. S. McFarlane commented that they had found the isotope-dilution method of great value in determining proteins in tissue homogenates. The essential part of the technique was to prepare beforehand an antiserum of the protein in question; labelled protein was homogenised with the tissue and a portion of the protein was recovered by specific precipitation and counted.

Dr. A. R. Somerville asked the author if he had had any experience of spontaneous chemical decomposition of labelled compounds.

Dr. Lester Smith replied that he had not had any experience of this phenomenon.

Dr. R. F. Glascock remarked that it had been stated at the Radio-isotope Conference at Oxford in 1954 that some compounds were more labile than others and that, in particular, [^{35}S]methionine was subject to radiolysis. He had been told that water containing about 20 per cent. of tritium oxide underwent radiolysis to hydrogen and oxygen. They had some tritium-labelled hexoestrol of specific activity about 20 μC per μg, which they were watching carefully for radiolysis.

Bioassay of Radio-iodinated Plasma Proteins for Clinical Use

Dr. A. S. McFarlane stated that labelled plasma proteins were now used in humans for measuring blood volumes, lymph volumes, cardiac efficiencies and in the diagnosis of placenta praevia and in general metabolic investigations. For some of these purposes it was only necessary that the "label" should be firmly bound to the protein and that the labelled material should persist in the blood stream for a period of minutes. For other purposes it was essential that the iodinated protein should be indistinguishable by the recipient from his own proteins over a period of weeks. It was not sufficient to show that the iodinated protein had the same sedimentation, electrophoretic and diffusion characteristics as the original, but the method of labelling should be subjected to a critical biological test involving comparison of *in vitro* labelled protein with biosynthetically labelled protein in the same animal. Carbon-14-labelled rabbit plasma proteins were particularly suitable for this purpose and their use had revealed serious deficiencies in some currently used methods of iodination. Iodinated human albumin could be compared with ^{14}C-labelled rabbit albumin in the same rabbit for 8 to 10 days before immunity developed. The two should give the same values for blood volume, mass ratio of lymph to plasma albumin (2·3 to 2·7), percentage survival at 100 hours (25 per cent.) and exponential elimination rate (8·2 per cent. per day).

Additional criteria of sterility, pH, radioactivity and freedom from radio-iodine not bound to protein had also to be satisfied.

Discussion

Dr. Watkins said that, although many papers described the use of ^{131}I-labelled insulin for the study of metabolism of the drug, it was still a matter for conjecture whether this method gave a true picture of insulin metabolism in the animal body. He would like to hear what comments Dr. McFarlane had to make on this, and whether he would expect ^{35}S-labelled insulin prepared by biosynthesis through methionine to give a more reliable picture.

Dr. McFarlane replied that the fact that plasma proteins could be iodinated in special conditions to behave like the un-iodinated proteins cannot be taken to mean that other proteins such as insulin could also be so iodinated. Until [^{131}I]insulin is shown to behave like [^{35}S]insulin the latter is undoubtedly the more reliable labelled substance.

Dr. W. Mulligan asked if the author had any information on the effect on proteins of labelling with iodine-131 by the method of R. C. Gilmore, M. C. Robbins and A. F. Reid (*Nucleonics*, 1954, **12**, 65).

Dr. McFarlane said that he had no information on the method.

Dr. W. F. R. Pover said that, in order to increase the reaction time for iodinating proteins, they had used a solution of iodine-131 in chloroform extracted after liberating free iodine-131 by oxidation of Amersham solution. The chloroform solution was shot through a fine jet into alkaline buffered protein solution, which was well agitated. The reaction then took place between two phases and was observedly slowed down. In experiments on man involving the use of ^{131}I-labelled protein and fats, they had always fed considerable quantities of inactive iodide to block the thyroid. He asked if Dr. McFarlane followed the same procedure with his animals. He pointed out that some of the experiments described by Dr. McFarlane, illustrating the rate of mixing of labelled protein injected into the rabbit, showed that the first mixing stage took 4 to 5 days, and he asked if Dr. McFarlane really believed that it took as long as that for active proteins in the serum to mix with the protein in the lymph space.

Dr. McFarlane said that any step that delayed the reaction between protein and iodine should help to achieve a desirable even distribution of the label. In contrast, chloroform tended to form a gel with plasma proteins, which might be disadvantageous. As a routine, they protected the thyroid in animal and human investigations by giving inactive iodine in the diet. The long delay in achieving equilibrium between lymph and plasma was a surprising finding, but could be explained if plasma proteins that passed out of the capillaries into the lymph were obliged to return exclusively by a tortuous route via the lymphatics and the thoracic duct.

Dr. J. F. Loutit commented on the slide showing the "decay" of labelled albumin and globulin in the circulation of man. These graphs showed clearly the early fall to one-third of the original figure, attributed to dilution of these proteins in the lymph. But, according to the graph, the urinary excretion of iodine-131 label was also excessive in the first few days. He asked how this was explained, other than by a non-exponential destruction of the labelled protein owing to denaturation or a similar effect.

Dr McFarlane replied that, if labelled plasma protein rather than lymph protein was the immediate metabolic precursor of urinary iodine-131, they would expect to find the close similarity in shape of plasma specific activity and urinary excretion curves shown on the slide. The possibility that any substantial part of the injected material was denatured in the sense that the label was promptly stripped off and excreted in the urine was excluded by the following findings. After six days the plasma specific-activity curve was truly exponential, corresponding to a turn-over of 3·1 per cent. per day. Therefore in the first six days approximately 17 per cent. of the injected protein must have been catabolised, the total urinary excretion of the label in the same period being 24 per cent. Of the 7 per cent. excess, not more than a quarter could be attributed to free iodide in the injection, while nearly all of the remainder could be due to iodine-131 liberated from fibrinogen. The breakdown of this protein was so rapid that it did not significantly affect the turn-over measured after six days, *i.e.*, the turn-over rate of 3·1 per cent. per day was sensibly that of total serum proteins. However, the iodine-131 liberated from the fibrinogen all appeared in the urine in the first few days. There was thus little activity in the urine that could be attributed to denatured protein.

The problem posed by Dr. Loutit could also be looked at in another way. During the first 24 hours, when on an average 80 per cent. of the injected protein was still in the blood stream, approximately 8 per cent. of the injected label appeared in the urine. The true metabolic rate of 3·1 per cent. per day applied to *all* the protein—lymph and plasma—and there was approximately twice as much protein in the lymph as in the plasma. If 80 per cent. of the injected label was in a pool only one-third of the size of the lymph and plasma pool, the true metabolic rate for protein in the plasma would be 80 per cent. of 9·3 per cent., or 7·5 per cent., of the dose injected, which agreed satisfactorily with the urinary value of 8·0 per cent.

Isotope-dilution Assay of Antibiotics in Fermentation Liquors with Particular Reference to Benzylpenicillin and Griseofulvin

Mr. G. C. Ashton said that, because of interference from other penicillins and from benzylpenicillin precursors, the quantitative determination of benzylpenicillin in fermentation-broth samples by conventional analytical procedures was extremely difficult.

Isotope-dilution assay became the method of choice for such complex mixtures because quantitative isolation of the benzylpenicillin was not necessary. Isotope-dilution assay procedures for benzylpenicillin based on the stable isotopes carbon-13 and deuterium had been described, but the preparation of the labelled benzylpenicillin was costly, and so was the apparatus required for measuring the isotope dilution. A procedure involving ^{14}C-labelled benzylpenicillin had been described (G. C. Ashton and M. C. Foster, *Analyst*, 1955, **80**, 123). This had the advantage that both the preparation of the "label" and the equipment required were relatively inexpensive. A further advantage of using radioactive-labelled materials for isotope-dilution assays was that the precision of the result was not limited by the instrumentation, as it was with stable isotopes.

Isotope-dilution assay had also been applied to the determination of griseofulvin in fermentation samples, ^{36}Cl-labelled griseofulvin being used, not because of the complexity of the mixture, but to provide an independent check on a newly developed spectrophotometric procedure for griseofulvin in the absence of alternative methods (G. C. Ashton, A. P. Brown and J. P. R. Tootill, *Analyst*, 1956, **81**, 220, 225 and 228).

They preferred to use end-window counting for both ^{14}C- and ^{36}Cl-labelled samples, using, generally, 1-sq.cm polythene planchettes. They had observed that "infinite-thickness" counting was subject to quite large errors with this type of planchette unless the weight of the planchette contents was carefully controlled. For example, with ^{14}C-labelled phenylacetic acid a 1-mg difference in the weight of the planchette contents at the so called "infinite thickness" level would give a mean count difference of about 0·4 per cent. With ^{36}Cl-labelled griseofulvin it was necessary to adjust the weight on the planchette to within $\pm$ 0·1 mg. It was possible that the disappointing results obtained by some workers with this type of counting might be due to lack of appreciation of the necessity for careful control of the weight of the planchette contents.

The accuracy and precision of isotope-dilution assays with radioactive tracers had been discussed previously (G. C. Ashton and M. C. Foster, *Analyst*, 1955, **80**, 123). To obtain an accurate result, *i.e.*, a result free from bias, it was essential that the isolated material counted on the planchette should be pure. The quantity of labelled material added to the broth samples must be accurately known. This knowledge was most accurate when the labelled material was itself demonstrably pure. An accurate result would only be obtained if the isotope-dilution measurement was properly carried out (in this instance with particular reference to the weights on the planchettes and with due care in preparing the planchettes). The labelled material itself (or its degradation product, if this was to be counted) must not be so highly radioactive that counts were lost when counting the label but not when counting the diluted mixture.

The precision (*i.e.*, "spread" of the results) of a radioactive isotope-dilution assay was dependent on the total counting time, on the proportion of this time allocated to label count, diluted-label count and background count, and on the ratio of the weight of the material to be assayed in the sample to the added label weight.

With due regard to detail it was possible to obtain isotope-dilution assay results with a standard error of $\pm$ 2 per cent. If the precision expected from the counting data was not reflected in the repeatability of replicate assays on the same sample, the accuracy of the results, and not their precision, must be suspect.

Discussion

Dr. R. F. Glascock said he was interested to see that the "infinitely thick" samples described by Mr. Ashton were not infinitely thick; he asked how the author accounted for this. One of the tables shown had indicated that the counting rate of "infinitely thick" samples continued to increase with thickness; he wanted to know if the weights shown were per sq. cm or per disc. He asked what sort of agreement was obtained between replicate platings of the same radioactive sample.

Dr. W. F. R. Pover said that they, too, had found this apparent "infinite thickness" that increased in counting rate with increase in thickness. They had attributed the effect to the fact that these samples

were usually counted as close to the end-window counter as possible, and that in increasing the "thickness" (weight) one was also increasing the physical thickness and bringing the sample closer to the tube, thereby increasing the solid angle counted ("geometry"). There was also an edge effect, which would require either an infinitely wide sample or, perhaps, a sample formed in the shape of part of a sphere, to remove the effect, as shown in Fig. 1.

MR. ASHTON replied to Dr. Glascock that the weights shown were per sq. cm and per disc, a 1-sq. cm planchette having been used. It was just because the counting rate of apparently infinitely thick samples on this type of planchette did increase with increasing weight that they had found it necessary to control the weight of sample to obtain statistically consistent results.

Provided the weight difference between the replicate planchettes was not large enough to cause a bias greater than the error due to the precision of the individual counts, then in their experience the replicate counts would agree when tested by the χ^2 test.

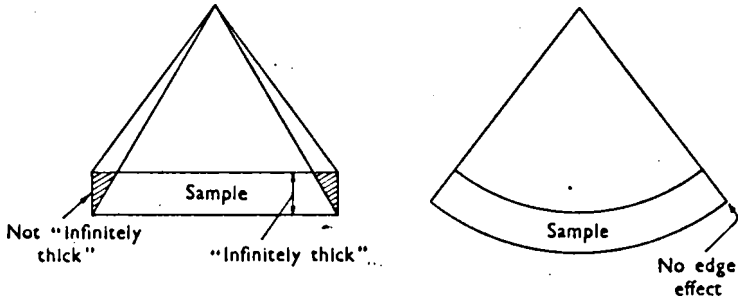


Fig. 1. Possible method of overcoming edge effect

He could not emphasise too strongly the need to control the weight of the planchette contents if counts were to be consistent.

MR. ASHTON said he thought that Dr. Pover's suggestion for the increase in count with increasing weight at the "infinite thickness" level seemed logical. Rather than change the geometry of the counting equipment it would seem that careful control of the weight and physical form of the sample would remove the effect.

THE ASSAY OF ALDOSTERONE AND OTHER ADRENAL STEROIDS BY THE $^{24}Na/^{42}K$ METHOD

DR. J. F. TAIT, presenting this paper by R. N. Jones, Sylvia A. Simpson and himself, said that aldosterone, an adrenal hormone that had been isolated, identified and synthesised in the last two years, had a potent action on mineral metabolism. Its isolation was facilitated by the use of a bioassay method that measured the effect of adrenal hormones on the urinary $^{24}Na/^{42}K$ ratio of adrenalectomised rats after the injection of a mixture of the isotopes. These two isotopes emitted β-rays of differing energy and hence their ratio in a mixture could be measured by a simple single-filter technique.

The method had recently been re-examined with the object of finding the most suitable conditions for determining aldosterone in biological fluids. Log $^{24}Na/^{42}K$ and log (dose of aldosterone) had been demonstrated to be the most suitable response and dose metameters, and the superiority of the use of $^{24}Na/^{42}K$ rather than ^{24}Na or ^{42}K singly had been established. No superiority of the use of the radioactive ratio over the inert ratio of sodium and potassium has been shown and hence the choice of the use of isotopes in this connection was one of individual convenience. As little as 0·1 μg of aldosterone could be detected ($P = 0{\cdot}01$) and 1 μg determined with fiducial limits of approximately 20 per cent. ($P = 0{\cdot}05$) by using the radioactive bioassay method.

Dr. Tait also described a method for the determination of aldosterone and cortisol in human urine by an isotope-dilution technique. In this, 2 μg of [4-^{14}C]cortisol were added to the urine and, after extraction and purification stages in which cortisol and aldosterone had identical properties, the resulting mixture was acetylated. Then 0·5 μg of [*carboxy*-^{14}C]aldosterone diacetate was added and the acetyl derivatives of cortisol and aldosterone were separated by column chromatography. The specific activities of the derivatives were measured, and then the initial quantities present in urine could be calculated. By this method 1 μg of aldosterone could be determined with a coefficient of variation of about 10 per cent. The physico-chemical and biological methods were therefore of equal precision.

The latter method had been presented as an example of an isotope-dilution technique in which, although it was necessary that the added radioactive compounds be pure, it was not essential that the final products be crystalline, provided that specific methods were used for their determination. The advantages of such a method were considered to be—

1. Recovery values were obtained for every individual analysis.

2. As the amount of added labelled compound was very small, the estimates of recovery were more valid than those arrived at by measuring the recovery of relatively large amounts of added material.

3. When column chromatography was used for subsequent purification, much labour could be saved by restricting analysis to the appreciably radioactive fractions.

4. By correlating radioactivity with the estimate of total material in the fractions of the column, the most appropriate and specific analytical method could be chosen with speed and convenience and, if necessary, the specificity of the reaction could be tested in a similar manner for every determination. This procedure seemed to be particularly useful when fluorescence methods were being examined.

It was concluded that in this particular field the use of radioactive isotopes in the bio-assay method had proved of value in the past, but with the increasing availability of reliable flame photomers would be less so in the future. It was the opinion of the authors, however, that the use of isotope-dilution methods, as described, could greatly facilitate the analysis of micro quantities of compounds in biological fluids and in particular that of steroids such as the adrenocortical hormones and the natural oestrogens and androgens.

Assay of TSH Based on the Rate of Discharge of Radioactive Iodine from the Thyroids of Chicks

Dr. Trevor Kinnear described a procedure in which day-old cockerels were injected with iodine-131 and for the succeeding 3 to 4 days received a daily injection of sodium L-thyroxine. Determination of the radioactivity in the region of their thyroids was then carried out *in vivo*, and repeated 48 hours later, after the chicks, in groups of 10, had received saline (controls) and different concentrations of a standard TSH preparation and of the test material. The percentage discharge of radio-iodine during this period was calculated for each group, and the amount discharged by the treated groups in excess of that lost by the controls provided a measure of thyrotrophic activity.

Labelled Metabolic Pools for Studying Quantitatively the Biochemistry of Toxic Action

Mr. F. P. W. Winteringham said that a biochemical lesion of toxic action may be regarded not so much as an enzyme interference, but as an accumulation or a depletion of a metabolite beyond the tolerance limits of the healthy tissue. A method of over-all analysis of the metabolic pools involved in the minute-to-minute functions of the tissues from poisoned and from control animals would therefore provide a powerful tool for studying acute toxic mechanisms in the intact animal. This had been achieved in insects by feeding or injecting them with suitably labelled substrates, when well defined metabolic pools became rapidly labelled. The tissues were then extracted under conditions that precluded further chemical or enzymic action and the labelled metabolites so extracted were resolved by uni-dimensional paper chromatography. The labelled metabolites separated on the resulting paper chromatograms were then located and determined by scanning with a suitable Geiger - Müller counter (F. P. W. Winteringham, A. Harrison and R. G. Bridges, *Analyst*, 1952, **77**, 19; A. Harrison and F. P. W. Winteringham, *Nucleonics*, 1955, **13** [3], 64). In this way a radiochromatogram was obtained showing what might be described as a spectrum of the labelled metabolites. Labelled fractions that contained more than one compound and which were not resolved on a first chromatogram were re-concentrated on a fresh strip of paper by a simple elution technique and then re-chromatographed in an alternative solvent (F. P. W. Winteringham, *Nature*, 1953, **172**, 727). This procedure might be repeated, so that the resolving powers of multi-dimensional chromatography could be exploited, but the separated compounds could always be scanned on uni-dimensional strips, a considerable advantage in quantitative radioactive tracer - paper chromatography. These techniques had been successfully applied

to the analysis of the soluble phosphorus pool of the tissues of the adult housefly, *Musca domestica* L., and to the study of the effects of various insecticides thereon. The striking similarities between the phosphorus metabolism found in insects and in mammals suggested, however, that the results obtained were likely to be of significance in mammalian toxicology. The principal fractions identified in the housefly tissues were adenosine triphosphate, diphosphate and monophosphate (ATP etc.), argininephosphoric acid (Arg-P), glucose 6-phosphate (G6-P), phosphoglycerate (PGA) and inorganic phosphate (PO_4''') (F. P. W. Winteringham, R. G. Bridges and G. C. Hellyer, *Biochem. J.*, 1955, **59**, 13). It was shown that under the experimental conditions used the α-, β- and γ-phosphorus atoms of the ATP were equally labelled. This was demonstrated in two ways: by acid hydrolysis of the ATP, which yielded ribose 5-phosphate containing one-third of the total phosphorus-32 activity of the parent ATP, and by neutron activation of the paper chromatograms, in which the original phosphorus-32 activity had decayed away. The latter technique permitted the relative specific phosphorus-32 activities of the original fractions to be estimated and the results indicated that these were uniform. Brief exposure (60 seconds) of flies that had been fed with $[^{32}P]PO_4'''$ to methyl bromide, an established SH-enzyme inhibitor, brought about a spectacular reduction in tissue ATP, the entire phosphorus of the ATP sometimes appearing as PO_4'''. Longer exposure (1 hour) caused depletion of PGA, an expected consequence of SH-enzyme inhibition and of the inhibition of triose phosphate dehydrogenase in particular. Similar exposure to ethylene dibromide, a suspected SH-enzyme inhibitor, also depleted PGA but was without effect on ATP levels. Deep narcosis induced by brief exposure of the flies to ethylene dichloride or to *cyclo*propane was without significant effect on the phosphorus distribution (F. P. W. Winteringham and G. C. Hellyer, *Biochem. J.*, 1954, **58**, xlv).

Many intermediates of oxidative metabolism, protein, fat synthesis and so on did not contain phosphorus and could not therefore be studied by labelling the phosphorus pool. These studies were therefore extended by labelling metabolic pools with carbon-14. Carbon-14-labelled acetate was injected into individual houseflies as the primary substrate on account of its central metabolic role. Labelled flies were finally homogenised, extracted and the labelled metabolites resolved and assayed by the methods described. Principal ^{14}C-labelled soluble fractions had been identified as glutamic acid, proline and glutamine, which were present in the free state in remarkably high concentrations in the insect blood. A minor fraction had been tentatively identified as acetylcholine, which could thus be assayed with a greater sensitivity and specificity than by conventional pharmacological techniques. An interesting result of some preliminary work was that exposure of the insects to the potent anticholinesterase di*iso*propyl phosphorofluoridate (DFP) apparently failed to cause any sustained increase in acetylcholine but did cause a substantial increase in the free glutamine. This had interesting implications on account of the known role of glutamine in ammonia detoxication, and the suspected role of ammonia in convulsions, which were also observed in the DFP-treated insects.

Much of the data quoted has been the result of team work, particularly by the author and his colleagues Messrs. A. Harrison and G. C. Hellyer.

Discussion

Mr. Winteringham, in reply to Dr. Pover, said that no special precautions had been taken to remove trace elements from the paper before radioactivation. Whatman No. 1 paper was used. It was washed by soaking successively in very dilute hydrochloric acid, aqueous ammonia and glass-distilled water.

There was a high soft-beta background, but the effects of this were readily eliminated by scanning through a 30-mg per sq. cm screen.

Replying to Dr. Glascock, Mr. Winteringham said he felt that the uniform labelling of the insect ATP under the experimental conditions used was probably due to a higher turn-over rate in the insect rather than to a different mechanism. It seemed likely that the α-phosphorus atom of vertebrate ATP would also become labelled if given sufficient time, but no one seemed to have done this with intact animals.

The Use of ^{131}I-labelled Serum Albumin in Determining the Intercellular Plasma in Centrifuged Red Cells

Dr. W. Mulligan, who presented this paper by F. W. Jennings, I. M. Lander and himself, said that blood samples were treated with a small volume of homologous serum albumin trace-labelled with iodine-131. After thorough mixing, centrifugation was carried

out in polythene tubes. Most of the supernatant plasma was removed and the cell column was frozen by plunging the tube into a solid carbon dioxide - acetone mixture. The frozen cell column was then divided into 1-cm sections, whose volume was determined gravimetrically. The cell samples and aliquots of supernatant plasma were diluted to 10 ml and radioactivity determinations were made in a liquid counter. It was possible from this to calculate the total amount of plasma trapped in the red-cell column and to study the distribution of intercellular plasma at different heights up the cell column.

After centrifugation at an r.c.f. (relative centrifugal force) of 1500 ***g*** for 30 minutes, ox and sheep bloods showed a much higher value for intercellular plasma than those of horse, rabbit, dog or pig. Prolonged centrifugation (3 hours) was found to be necessary with sheep and ox bloods to give figures comparable to those obtained for the other species. During these investigations it was observed that the temperature at which centrifugation was carried out had an important influence on the magnitude of the intercellular plasma, *e.g.*, the percentage of trapped plasma in ox blood determined at 15° C was found to be $11{\cdot}9 \pm 3{\cdot}9$; at 25° C under the same conditions the figure was $6{\cdot}1 \pm 1{\cdot}3$.

The conditions chosen finally for the determination of intercellular plasma were (*a*) an r.c.f. of 1500 ***g***, (*b*) a temperature of 25° C, and (*c*) a centrifugation time of 3 hours for ox and sheep bloods, 1 hour for the other species studied. The results obtained under these conditions are summarised in Table I.

TABLE I

INTERCELLULAR PLASMA

R.C.F.: 1500 ***g***. Temperature: 25° C.

Species	Number of determinations	Time of centrifugation, hours	Trapped plasma, %	Range
Ox	28	3	$6{\cdot}1 \pm 1{\cdot}26$	3·2 to 7·8
Sheep	13	3	$4{\cdot}0 \pm 0{\cdot}46$	3·3 to 4·6
Pig	21	1	$4{\cdot}1 \pm 0{\cdot}26$	3·7 to 4·7
Horse	10	1	$2{\cdot}7 \pm 0{\cdot}22$	2·4 to 3·1
Dog	13	1	$4{\cdot}0 \pm 0{\cdot}28$	3·5 to 4·5

DISCUSSION

DR. MAGNUS PYKE asked if the author had determined the dry-matter content of the centrifuged cells. In yeast, the moisture content of the cell was affected by the temperature of propagation. Conway had shown that the moisture content of yeast cells was composed of three fractions: extracellular moisture, labile intracellular moisture and moisture that was an essential part of the cell structure. He asked if these observations had any relevance for blood cells.

DR. MULLIGAN replied that there did not appear to be much similarity between the two systems. Since the labelled albumin did not penetrate into the red cells, what they were measuring was purely extracellular fluid.

MR. G. C. ASHTON asked if determinations of trapped plasma had been carried out in which the cells had been allowed to settle under gravity instead of centrifuging. He asked if it was possible that distortion of cells might be responsible for irregularities in the results for bovine blood.

DR. MULLIGAN said that he did not know of any determinations of trapped plasma in which the cells had been allowed to settle without centrifugation. It seemed likely that some degree of distortion must always occur if the cells were packed so closely that the intercellular plasma represented only about 4 or 5 per cent. of the total packed-cell volume.

THE MEASUREMENT OF HEALTH HAZARDS

DR. J. F. LOUTIT gave a general review of the dangers associated with the type of work reported in the earlier papers and described the precautions that were necessary.